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Alveolar macrophages modulate allergic inflammation in a murine model of asthma

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Abbreviations: AM, alveolar macrophage; BAL, bronchoalveolar lavage; LPS, lipopolysaccharide; OVA, ovalbumin

Abstract

The role of alveolar macrophages (AMs) in the pathogenesis of asthma is still unknown. The aim of the present study was to investigate the effects of AM in the murine model of asthma. AMs were selectively depleted by liposomes containing clodronate just before allergen challenges, and changes in inflammatory cells and cytokine concentrations in bronchoalveolar lavage (BAL) fluid were measured. AMs were then adoptively transferred to AM-depleted sensitized mice and changes were measured. Phenotypic changes in AMs were evaluated after *in vitro* allergen stimulation. AM-depletion after sensitization significantly increased the number of eosinophils and lymphocytes and the concentrations of IL-4, IL-5 and GM-CSF in BAL fluid. These changes were significantly ameliorated only by adoptive transfer of unsensitized AMs, not by sensitized AMs. In addition, *in vitro* allergen stimulation of AMs resulted in their gaining the ability to produce inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, and losing the ability to suppress GM-CSF concentrations in BAL fluid. These findings suggested that AMs worked probably through GM-CSF-dependent mechanisms, although further confirmatory experiments are needed. Our results indicate that the role of AMs in the context of airway inflammation should be re-examined.

Keywords:

adoptive transfer; airway inflammation; alveolar macrophage; asthma; GM-CSF

Introduction

Alveolar macrophages (AMs) are the most abundant cells in the alveolar spaces and conducting airways, and are known to be involved in immune homeostasis in the respiratory tract (Wissinger *et al.*, 2009). Asthma is a disease characterized by immune-mediated airway inflammation. Several reports have suggested that AMs suppress allergen-specific immune response and airway inflammation (Thepen *et al.*, 1991, 1992; Tang *et al.*, 2001; Careau *et al.*, 2010). However, this property seems to be dependent on the functional status of AMs. Sensitized AMs promote eosinophilic airway inflammation (Moon *et al.*, 2007) and take part in acute asthma exacerbation by stimulating CD4-positive T cells to secrete cytokines (Herbert *et al.*, 2010). A recent report has shown that allergen sensitization modulates AM function and only unsensitized AMs protect against development of airway hyperresponsiveness (Careau *et al.*, 2006).

In this study, we compared the effects of sensitized and unsensitized AMs on the reduction of allergic inflammation and cytokine secretion in a murine model of asthma. Phenotype changes were first evaluated after selective depletion of AM populations with liposomes containing clodronate (dichloromethylenediphosphate or Cl_2MDP). Then, sensitized or unsensitized AMs were adoptively transferred into AM-depleted sensitized mice and numbers of inflammatory cells and cytokine concentrations in bronchoalveolar (BAL) fluid were measured. Finally, we evaluated the phenotypic changes in AMs after *in vitro* allergen stimulation.

Results

Changes in inflammatory cells and cytokine levels in BAL fluid after alveolar macrophage depletion

Administration of clodronate (Cl_2MDP)-encapsulated

liposomes resulted in significant increases in total cell, eosinophil, and lymphocyte counts in BAL fluid (Figure 1). Cytokine concentrations in BAL fluid, IL-5 and GM-CSF, showed significant increases after AM-depletion (Figure 2). IFN-γ and IL-13 (data not shown) concentrations showed no differences and the levels of IL-12p70 were below the detection limit.

Changes in inflammatory cells and cytokine levels in BAL fluid after adoptive transfer of sensitized or unsensitized alveolar macrophages to alveolar macrophage depleted sensitized mice

When sensitized AMs were adoptively transferred to AM-depleted sensitized mice, there were no differences in BLA fluid inflammatory cell counts. However, adoptive transfer of unsensitized AMs significantly reduced total cell, eosinophil, and lymphocyte counts in BAL fluid (Figure 1). Similarly, adoptive transfer of sensitized AMs did not significantly decrease the concentrations of inflammatory cytokines in BAL fluid. However, adoptive transfer of unsensitized AMs significantly reduced the concentrations of GM-CSF in BAL fluid (Figure 2). Macrophages recruited from regional tissue or circulation (Moon *et al.*, 2007) after intranasal challenge of OVA may explain the insignificant difference in the number of macrophages shown in Figure 1.

Pro-inflammatory cytokine production from alveolar macrophages after *in vitro* OVA stimulation

AMs obtained from unsensitized mice expressed little or nopro-inflammatory cytokines (IL-1β, IL-6, and TNF-α), but AMs obtained from sensitized mice produced significantly higher concentrations of pro-inflammatory cytokines after *in vitro* OVA stimulation (Figure 3).

Discussion

In the present study, we demonstrated that AM-depletion after sensitization significantly increased the number of eosinophilis and lymphocytes, and the concentrations of IL-4, IL-5, and GM-CSF in BAL fluid in a murine model of asthma. Moreover, increased numbers of eosinophilis and lymphocytes and concentrations of GM-CSF in BAL fluid after AM-depletion were significantly ameliorated

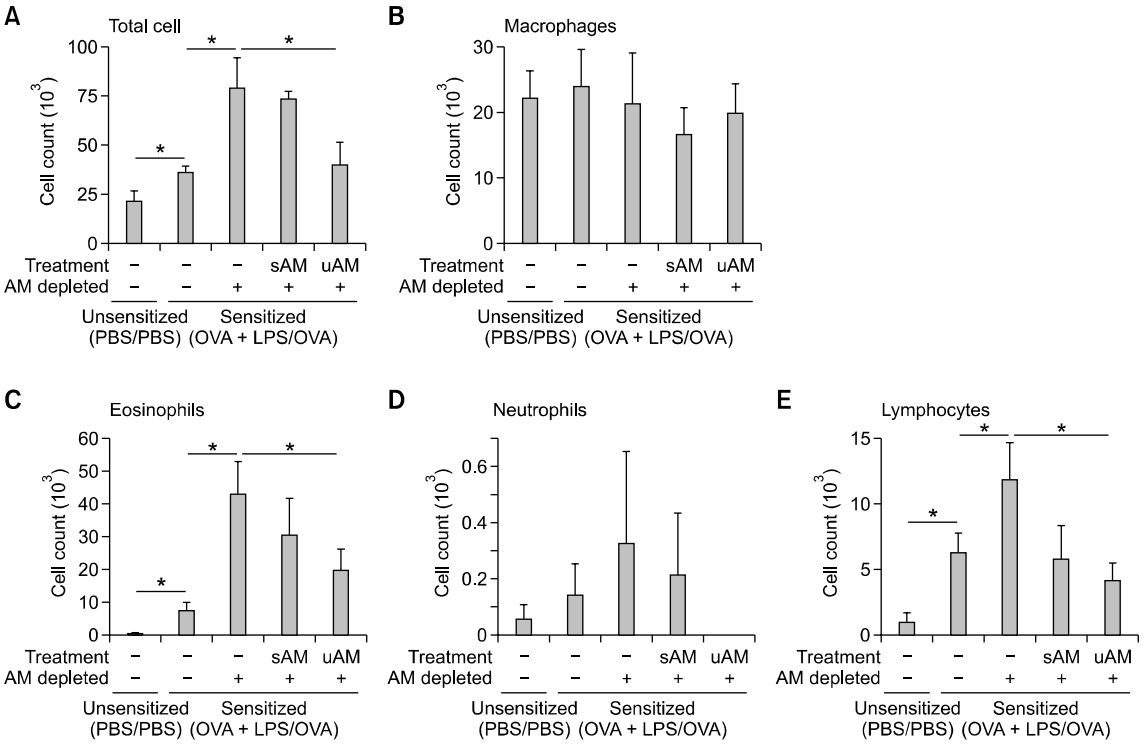

Figure 1. Changes in inflammatory cells in BAL fluid after alveolar macrophage depletion and after adoptive transfer of sensitized or unsensitized alveolar macrophage to alveolar macrophage depleted mice (A) total cell, (B) macrophages, (C) eosinophils, (D) neutrophils, (E) lymphocytes, LPS, lipopolysaccharide; sAM, sensitized alveolar macrophages; uAM, unsensitized alveolar macrophages; OVA, ovalbumin, *, $P < 0.05$)

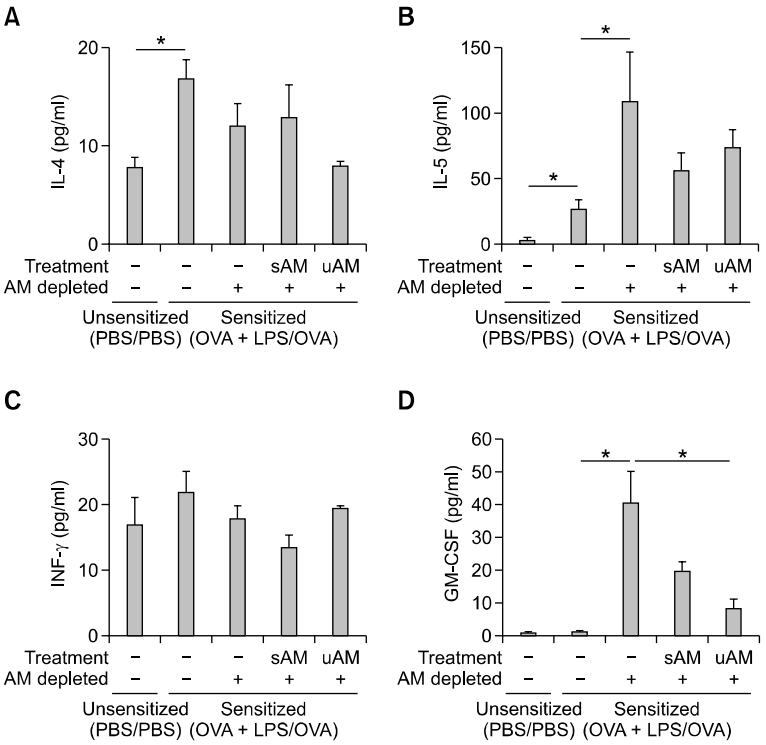

Figure 2. Changes in inflammatory cytokine levels in BAL fluid after alveolar macrophage depletion and after adoptive transfer of sensitized or unsensitized alveolar macrophage to alveolar macrophage depleted mice (A, IL-4; B, IL-5; C, IFN-γ; D, GM-CSF; LPS, lipopolysaccharide; sAM, sensitized alveolar macrophages; uAM, unsensitized alveolar macrophages; OVA, ovalbumin; *, $P < 0.05$)

only by adoptive transfer of unsensitized AMs, not by sensitized AMs. The observations, in conjunction with a previous report that unsensitized AMs can protect against development of airway hyperresponsiveness (Careau *et al.*, 2006), suggest that AMs may have an important role in the development of asthma. In addition, we believe that the gain in ability to produce inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, and the loss of ability to suppress GM-CSF concentrations in BAL fluid may contribute to the inability of sensitized AMs to play a protective role.

It was suggested that upon exposure to allergen, lung macrophages can convert to a Th1-oriented antigen presenting capacity by an IFN-γ-dependent mechanism, and that the participation of these cells in antigen presentation inhibits, to some degree, the development of Th2 immune responses in the bronchial mucosa (Tang *et al.*, 2001). Along with previous reports, we found that populations of eosinophils and lymphocytes in BAL fluid significantly increased after AM-depletion. However, we could not observe any significant change in IFN-γ concentrations in BAL fluid after AM-depletion, whereas, the GM-CSF concentrations in BAL fluid

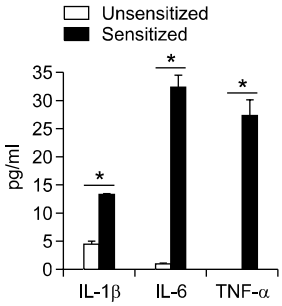

Figure 3. Pro-inflammatory cytokine production from alveolar macrophages after *in vitro* ovalbumin stimulation (*, $P < 0.05$).

changed significantly. This different observation may have been a result of differences in the mouse strain used and the experimental protocol, and detailed mechanisms for these findings should be investigated further. To the best of our knowledge, this is the first study showing that AMs may modulate allergic inflammation via GM-CSF in a murine model of asthma.

A recent report showed that the transfer of unsensitized AMs 24 h prior to OVA challenge

administered to AM-depleted, sensitized rats resulted in the abrogation of airway hyperresponsiveness, whereas the transfer of sensitized AMs to AM-depleted, unsensitized rats did not significantly alter airway responsiveness (Careau *et al.*, 2006). However, the authors did not observe any differences in the numbers of inflammatory cells in BAL fluid after AM depletion and transfer and concluded that the role of AMs in asthma is unrelated to reduction in inflammatory cell recruitment, but instead may involve other mechanisms modulating airway hyperresponsiveness. The observations are contrary to our findings. However, airway hyperresponsiveness generally correlates reasonably well with airway inflammation (Busse and Lemanske, 2001). AMs have long been recognized to be activated in asthmatic inflammation, with evidence of increased secretion of cytokines such as tumor necrosis factor α and IL-6 (Gosset *et al.*, 1991). In addition, it has been revealed that AMs from atopic asthmatic subjects, but not from atopic non-asthmatic subjects, enhance IL-4 and IL-5 production by $CD4^+$ T cells upon stimulation with allergen (Tang *et al.*, 1998a, 1998b). Recently, it was reported that the airway epithelial cell derived IL-33 amplified an alternative activation and chemokine productions from macrophages (Kurowska-Stolarska *et al.*, 2009). Taken together, it is clear that AM plays a potential role in the modulation of local inflammation.

However, in the present study using a murine model of asthma, we found that airway inflammation was not inhibited and even was increased after AM depletion. Similar findings were observed, in a rat model of trimellitic anhydride-induced occupational asthma (Valstar *et al.*, 2006). The authors reported that AM depletion augmented the trimellitic anhydride-induced tissue damage and inflammation 24 h after challenge. The reason for this phenomenon could be based on the functional status of the AMs. As mentioned previously, AMs play a key role in the maintenance of immunological homeostasis in the respiratory tract. As examples, allergen challenge of AM-depleted sensitized rats results in a highly elevated immunoglobulin E concentrations, large influxes of T and B cells into the lungs, and increase in airway hyperresponsiveness (Thepen *et al.*, 1991, 1992; Careau *et al.*, 2006). We also observed that inflammatory cells in BAL fluid significantly increased when AMs were depleted in mice in a murine model of asthma, and these increases were attenuated by the adoptive transfer of AMs, although significant changes were found only when unsensitized AMs were transferred. This suggest that unsensitized AMs are capable of reducing allergic inflammation, but they lose this ability as they become sensitized after repeated exposures to allergen. The inflammatory phenotypic that we observed in the present study maybe one of the reasons for this finding.

In summary, AM depletion significantly increased the numbers of total inflammatory cells, eosinophils and lymphocytes in BAL fluid, and adoptive transfer of unsensitized AMs effectively abrogated these change, probably through GM-CSF dependent mechanisms. Our results indicate the role of AMs in the context of airway inflammation should be re-examined.

Methods

Mice

Female C57BL/6 mice (6-week old) were purchased from the Jackson Laboratory (Bar Harbor, ME). All animals were maintained in filter-top cages under specific pathogen-free conditions at the animal facility of the Seoul National University College of Medicine (Seoul, Korea). Experimental procedures were performed with the approval of the Seoul National University Institutional Animal Care and Use Committee (IACUC) in accordance with the guidelines of IACUC.

Experimental protocols for a murine model of asthma and alveolar macrophage depletion

For sensitization, mice were anesthetized and ovalbumin (OVA, Sigma Chemical Co., St. Louis, US) plus 0.1 μg of lipopolysaccharide (E. coli O55:B5, Calbiochem, San Diego, US) in 30 μl of phosphate buffered saline (PBS) were instilled intranasally on days 1, 2, 3, and 8. On days 7 and 8 after the last sensitization with OVA plus LPS (days 15, 16), mice were intranasally challenged with OVA only (Supplemental Data Figure S1A). For AM depletion, mice were first challenged with OVA plus LPS as described previously (Elder *et al.*, 2004), and then received 30 μl of clodronate (Cl_2MDP)-encapsulated liposomes (Roche Diagnostics GmbH, Mannheim, Germany) on 3 consecutive days (days 12-14) just before the first OVA challenge (Supplemental Data Figure S1B). Confirmation that more than 75% of alveolar macrophages were depleted without affecting other cell types were performed by counting the macrophages in BAL fluid from treated and untreated mice.

Isolation of alveolar macrophages and adoptive transfer

Sensitized AMs were obtained from lungs of sacrificed mice 7 days after the last sensitization (day 15 in Supplemental Data Figure S1A), and unsensitized AMs were obtained from lungs of sacrificed mice who did not undergo sensitization. The lungs were perfused via the right ventricle with 5 ml of PBS and excised and minced lung tissue was incubated for 15 min at 37°C in 0.5% EDTA. To

obtain a single cell, the minced tissue was grinded on the strainer with complete RPMI (cRPMI) that was supplemented 10% FBS, L-glutamine, sodium pyruvate, penicillin-streptomycin, and erythrocytes were lysed by treatment with ammonium chloride solution (StemCell technologies, Vancouver, Canada). The cells were washed twice with RPMI, and centrifuged at 1500 rpm for 10 min. We defined AMs as $CD11b^-$ $CD11c^+$ cells. To isolate $CD11c^+$ cells, auto MACS sorter (Miltenyi Biotec, Auburn, CA) with anti-CD11c antibody conjugated to magnetic beads were used in accordance with manufacturer's instructions. Isolated $CD11c^+$ cells were then stained with anti-CD11b to sort $CD11b^-$ $CD11c^+$ cells. The stained cells were gated into $CD11b^-$ $CD11c^+$ cells according to expressions of CD11b or CD11c molecule and sorted using the live sterile cell sorting system (FACSAria system, Becton Dickinson, San Jose, CA). The purity of $CD11b^-$ $CD11c^+$ cells was $>$ 97%. Isolated sensitized or unsensitized AMs (1×10^5 cells) were transferred intratracheally to AM-depleted sensitized mice on the day of the first OVA challenge (day 15, Supplemental Data Figure S1C). Mice were sacrificed on day 18, and lung inflammation was evaluated (Supplemental Data Figure S1C).

Bronchial alveolar lavage fluid collection and cytokine measurements

A total of 2 ml (1 ml$\times$2) of BAL fluid using PBS was obtained after tracheostomy. Cells were collected by centrifugation at 3000 rpm for 15 min at 4°C. The pellets were resuspended in 100 μl of PBS, and total cells counts were determined using a Neubauer's chamber (hemocytometer). To determine differential cell counts, cytocentrifugation (Cytospin 3, Shandon, UK) was used at 500 rpm for 5 min at room temperature. BAL cells on slides were stained with Diff-Quik (Sysmex Co., Japan) and after air-dry, fixed with a synthetic mounting medium (Histomount, Ted Pella Inc.). At least 300 cells were counted in each preparation. The numbers of macrophages, eosinophils, neutrophils and lymphocytes in BAL fluid were determined as described previously (Moon *et al.*, 2010). The concentrations of IL-12p70, IFN-γ, IL-4, IL-5, IL-13 and GM-CSF in BAL fluids were measured using the Bio-Plex cytokine assay in accordance with the manufacturer's instructions (Bio-Rad Laboratories, Inc., Hercules, CA). Briefly, prepared beads were added to the pre-wetted filter plate, and then samples were added and incubated for 30 min at room temperature. Attached cytokines were detected by incubating the plates for 30 min with detection antibodies. Streptavidin-PE was added to the filter plates, which were read on the Bio-Plex reader.

Characterization of sensitized and unsensitized alveolar macrophages

To compare sensitized AMs and unsensitized AMs, pro-inflammatory cytokines were evaluated after *in vitro* stimulation of the cells by OVA. AMs ($CD11b^-$ $CD11c^+$ cells) were isolated from unsensitized and sensitized mice (just before OVA challenge; day 15 in Supplemental Data Figure S1A). The cells were stimulated by OVA (100 μg/ml) for 3 days, and then the concentrations of IL-1β, IL-6 and TNF-α in cell culture supernatant were measured using the Bio-Plex cytokine assay.

Statistical analysis

All data are reported as the mean ± standard error (SE) of the means. A two-tailed Student *t*-test was used for analysis and differences were considered significant for $P < 0.05$.

Supplemental data

Supplemental data include a figure and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-43-5-02.pdf.

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