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Antigen sensitization modulates alveolar macrophage functions in an asthma model

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Careau, Éric, Léa-Isabelle Proulx, Philippe Pouliot, Annie Spahr, Véronique Turmel, and Élyse Y. Bissonnette. Antigen sensitization modulates alveolar macrophage functions in an asthma model. *Am J Physiol Lung Cell Mol Physiol* 290: L871–L879, 2006; doi:10.1152/ajplung.00219.2005.—We have previously demonstrated that adoptive transfer of alveolar macrophages from allergy-resistant rats to alveolar macrophage-depleted allergic rats prevents airway hyperresponsiveness development, suggesting an important role for alveolar macrophages in asthma pathogenesis. Given that ovalbumin sensitization can modulate alveolar macrophage cytokine production, we investigated the role of sensitized and unsensitized alveolar macrophages in an asthma model. Alveolar macrophages from unsensitized or sensitized Brown Norway rats were transferred to alveolar macrophage-depleted sensitized rats 24 h before allergen challenge. Airway responsiveness to methacholine and airway inflammation were measured the following day. Methacholine concentration needed to increase lung resistance by 200% was significantly higher in alveolar macrophage-depleted sensitized rats that received unsensitized alveolar macrophages compared with alveolar macrophage-depleted sensitized rats that received sensitized alveolar macrophages. Tumor necrosis factor levels in bronchoalveolar lavage fluid of sensitized rats that received unsensitized alveolar macrophages were significantly lower compared with rats that received sensitized alveolar macrophages. Interestingly, alveolar macrophages of unsensitized animals showed higher phagocytosis activity compared with alveolar macrophages of sensitized rats, suggesting that sensitization modulates alveolar macrophage phagocytosis function. Our data suggest an important role of allergen sensitization on alveolar macrophage function in asthma pathogenesis.

airway hyperresponsiveness; cytokine; phagocytosis

THE INCIDENCE OF allergic respiratory diseases increased dramatically over the last two decades. The genetic predisposition to develop asthma is now well recognized (20). Numerous studies have also shown the importance of inflammatory cells, such as mast cells, lymphocytes, eosinophils, and neutrophils, as well as Th2 type cytokines in asthma (6, 19, 32). Interleukin (IL)-4 and IL-13, two Th2 cytokines, are important in immunoglobulin (Ig) class switching to IgE, whereas IL-4 and IL-10 play a crucial role in Th2 cell commitment (9, 16, 50). In contrast, Th1 cytokines (interferon- γ and IL-12) protect against asthma development in children (48). Other cytokines, such as tumor necrosis factor (TNF), have been demonstrated to amplify the effect of asthmatic inflammation (29, 42). Recently, a role of regulatory T cells (Th3 cells, T_R cells, $CD4^+CD25^+$ cells, and

natural killer T cells) has been suggested in the control of asthma and allergy, but the specific mechanisms are still unknown (2).

Alveolar macrophages (AM) are predominant immune effector cells in the alveolar spaces and conducting airways and play a key role in the immunological homeostasis of the lung (8, 30, 43). Although they are well known to suppress T cell activation and antigen presentation activities of dendritic cells (22, 41), their role in asthma is still debated. Using the liposome-mediated macrophage suicide technique (47), we have demonstrated that AM depletion of allergen-sensitized animals leads to airway hyperresponsiveness after allergen challenge (7). These data suggest that AM are essential for the prevention of airway hyperresponsiveness development.

Brown Norway rats are genetically predisposed to develop allergic disease. Ovalbumin (OVA)-sensitized Brown Norway rats produce high levels of IgE and develop both early and late-phase airway responses after allergen challenge (17, 18, 24). Using this animal model of airway hyperresponsiveness, we have demonstrated that transfer of AM from allergy-resistant Sprague Dawley rats to AM-depleted Brown Norway rats reduced airway responsiveness to the level of Sprague Dawley rats in a dose-dependent manner (7). In contrast, transfer of AM from Brown Norway rats to Sprague Dawley rats did not modulate airway responsiveness. These data suggest that AM can protect against airway hyperresponsiveness development and that AM functions of Brown Norway rats may be altered. These experiments were done with allergen-sensitized animals, but the role of OVA sensitization on AM activities in this model has not been examined.

We hypothesized that unsensitized AM can protect against the development of airway hyperresponsiveness via the production of cytokines. To investigate the role of allergen sensitization on AM functions, AM from unsensitized or sensitized Brown Norway rats were transferred to AM-depleted OVA-sensitized rats 24 h before allergen challenge. Sensitized rats that received unsensitized AM had lower airway responsiveness to methacholine and airway inflammation than those that received sensitized AM, suggesting that OVA sensitization modulates AM activities. Indeed, phagocytosis, an important AM function, was higher in unsensitized AM compared with sensitized AM, demonstrating that sensitization alters AM functions.

METHODS

Animal sensitization and challenge with allergen. Male Brown Norway rats (BN/Ssn), 80 days old, were obtained from Harlan-

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Sprague Dawley (Indianapolis, IN). All animals were maintained in filter-top cages in virus/pathogen-free conditions. After 1 wk of acclimation, the animals were sensitized with OVA grade V (Sigma Chemical, St. Louis, MO) and Al(OH₃) (BDH Laboratory Supply, Poole, UK) or saline as we previously described (43). Later (3 wk), animals were challenged for 5 min with aerosolized OVA. The Laval University Animal Care Committee approved the experimental protocol in accordance with the guidelines of the Canadian Council on Animal Care.

AM transfer. In this study, four groups of saline (S) or OVA (O)-sensitized rats were used (Fig. 1). *Group 1* represents the negative and positive controls, whereas *group 2* was used to verify the effect of AM depletion using clodronate (Cl₂MDP; Sigma Chemical)-liposomes 17 days after sensitization (47). AM transfer (*groups 3 and 4*) was performed as previously described (7). Briefly, bronchoalveolar lavage (BAL) was done 20 days after sensitization, and 2×10^6 cells were transferred by intratracheal intubation to rats that had received Cl₂MDP-liposomes 3 days earlier using the same technique. Animals received AM from BAL of unsensitized rats (*group 3*) or sensitized rats (*group 4*) to verify the effect of AM repletion. Animals were allowed to recuperate for 24 h before being challenged with OVA (*day 21*). Airway responsiveness to methacholine and airway inflammation was measured 24 h later.

Measurement of airway responsiveness. Airway responsiveness was measured 24 h after OVA challenge as previously described (8). Briefly, animals were anaesthetized, intubated, and connected to a Quatra-T CombiChamber (Scireq, Montreal, QC, Canada) to measure lung resistance (R_L). Animals were exposed to doubling concentrations of aerosolized methacholine for 30 s with airflow of 6 l/min air, and R_L measurements were taken every min for 5 min. The peak value of R_L was measured after each concentration, and the challenge was stopped at 128 mg/ml. Methacholine concentration required to cause 200% increase in resistance, EC₂₀₀R_L, was calculated by interpolation of the concentration-response curve from an individual animal.

Inflammatory cells and mediators. Evidence of an inflammatory response was evaluated using the following three different parameters: cytokine levels in the BAL fluid, number and type of cells present in the BAL, and in vitro AM cytokine production. BAL was performed 24 h after OVA challenge with 4 ml of PBS followed by 46 ml. The first 4 ml were centrifuged, supernatant was kept at -80°C, and cytokine levels were measured within 2 days. Cells in the 4 and 46 ml were pooled and counted, and AM were purified by adherence and kept in culture in RPMI 1640 medium for 24 h to measure cytokine release. Cell-free supernatants were kept at -80°C and tested within 1 wk.

Cytokine measurement. ELISAs to measure cytokine levels were performed with OptEIA kits from BD Pharmingen (San Diego, CA) for TNF, IL-10, and IL-4, and from Biosource International (Camarillo, CA) for IL-12p40, IL-12p70, and IL-13. Sensitivity of the assays was 4 pg/ml for IL-10 and IL-4, 3 pg/ml for IL-12 (p40 and p70), 1.5 pg/ml for IL-13, and 15 pg/ml for TNF. When cytokine levels were lower than the detection limit, the value calculated was zero.

Phagocytosis assay. AM were isolated from BAL of *groups 1S, 1O, 3O, and 4O* (Fig. 1) performed 21 days after OVA sensitization. AM were incubated with 2 μm polystyrene fluorescent beads in a ratio of 10:1 (beads-cell) for 6 h. Phagocytosis was characterized by flow cytometry using an EPICS ELITE ESP cytometer (Beckman-Coulter,

Miami, FL) to determine the percentage of cells having phagocytosed beads.

Statistical analysis. Statistical analysis for cellular, cytokine, and airway hyperresponsiveness data was made using an ANOVA table followed by a Fisher's post hoc test to determine statistical signifi-

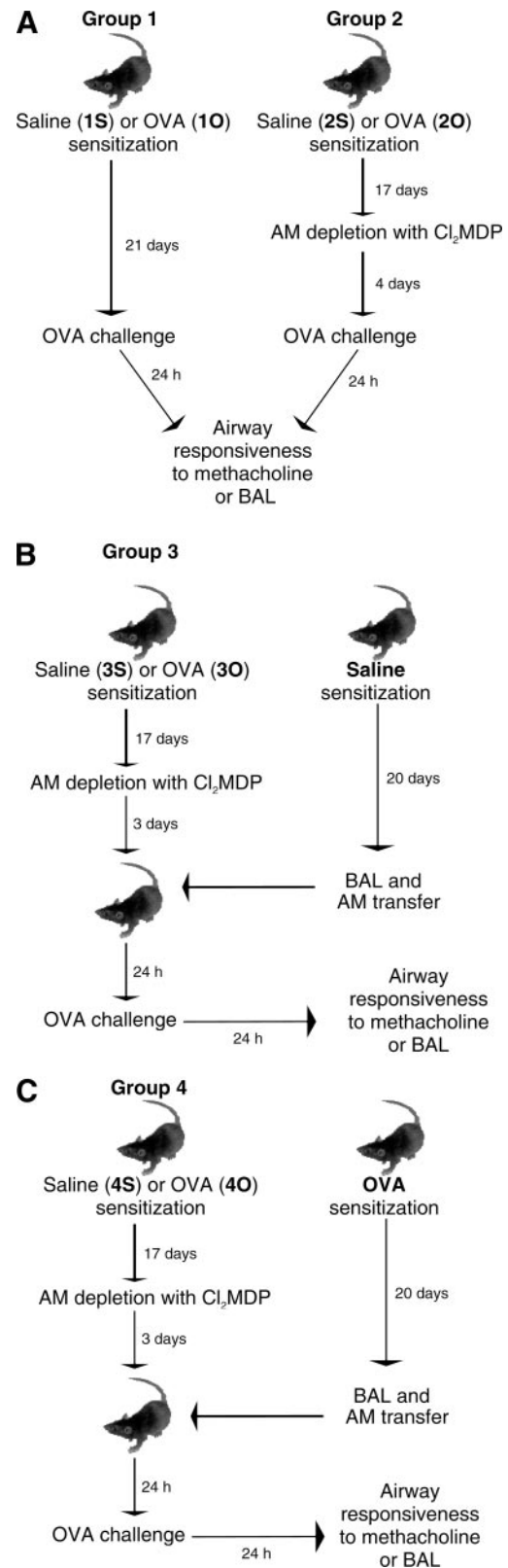


Fig. 1. Alveolar macrophage (AM) transfer protocol. A: AM from unsensitized (S) or sensitized (O) Brown Norway rats were depleted (*group 2*) or not depleted (*group 1*) before ovalbumin (OVA) challenge on *day 21*. B: AM from unsensitized Brown Norway rats were transferred to AM-depleted sensitized or unsensitized Brown Norway rats 20 days after OVA sensitization. C: AM from sensitized Brown Norway rats were transferred to sensitized or unsensitized AM-depleted Brown Norway rats 20 days after OVA sensitization. OVA challenge (for 5 min) was performed on *day 21*, and methacholine airway responsiveness or bronchoalveolar lavage (BAL) was performed 24 h later.



cance between groups (see Figs. 2–6). The Student's *t*-test for unpaired data was used to compare AM phagocytosis function. Differences were considered significant at $P < 0.05$.

RESULTS

Airway hyperresponsiveness. To investigate the role of antigen sensitization in the development of airway hyperresponsiveness, unsensitized (1S; Fig. 1) and OVA-sensitized (1O) rats were challenged with OVA, and airway responsiveness to methacholine was measured 24 h later. Furthermore, to determine the role of these cells in airway responsiveness, AM of unsensitized (2S) and sensitized (2O) rats were depleted. OVA challenge increased airway responsiveness (lower concentration of methacholine needed to obtain EC_{200R_L}) in sensitized (1O) rats (68.6 ± 5.9 mg/ml) compared with unsensitized (1S) animals (102.6 ± 8.9 mg/ml; Fig. 2). This increase was not significantly different when AM were depleted (2O, 59.8 ± 9.1 mg/ml). Furthermore, AM depletion after saline sensitization (2S) did not modify airway responsiveness. These data suggest that antigen sensitization, but not AM, is primordial in airway hyperresponsiveness development after allergen challenge in Brown Norway rats.

Although the presence of AM was not important in airway responsiveness of sensitized rats, we investigated whether unsensitized AM could modulate the development of airway hyperresponsiveness. The transfer of unsensitized AM to AM-depleted sensitized rats 24 h before OVA challenge abrogated airway hyperresponsiveness (3O vs. 4O; Fig. 2). In contrast, the transfer of sensitized AM to AM-depleted unsensitized rats (4S) did not significantly alter airway responsiveness of the later, suggesting that AM cannot transfer susceptibility to allergy. By themselves, the depletion and repletion of AM did not significantly modify airway responsiveness as observed in groups where we transferred unsensitized AM to AM-depleted

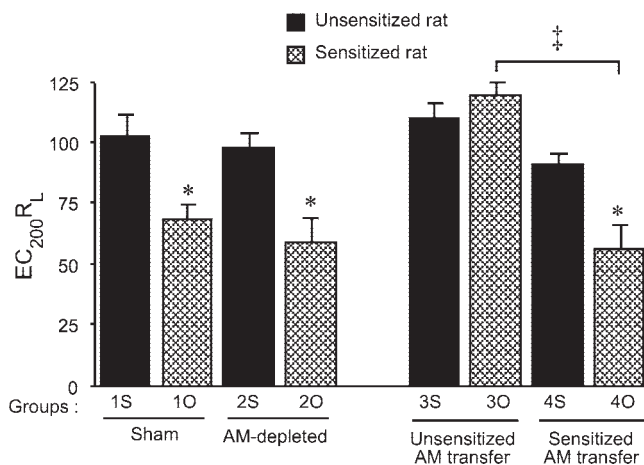


Fig. 2. Airway responsiveness of Brown Norway rats after OVA challenge. EC_{200R_L} represents the methacholine concentration needed to increase airway resistance by 200%. Sensitized Brown Norway rats with (1O) or without (2O) AM demonstrated significant ($P < 0.001$) airway hyperresponsiveness after OVA challenge compared with unsensitized rats (1S and 2S, respectively). Transfer of sensitized AM to sensitized animals (4O) did not modify the development of airway hyperresponsiveness, whereas transfer of AM from unsensitized rats to sensitized animals (3O) abrogated the OVA-induced reduced airway hyperresponsiveness ($P < 0.02$). Data are means $\pm$ SE of 5 rats. *Significant difference between unsensitized and sensitized rats. ‡Significant difference between unsensitized and sensitized AM transfer.

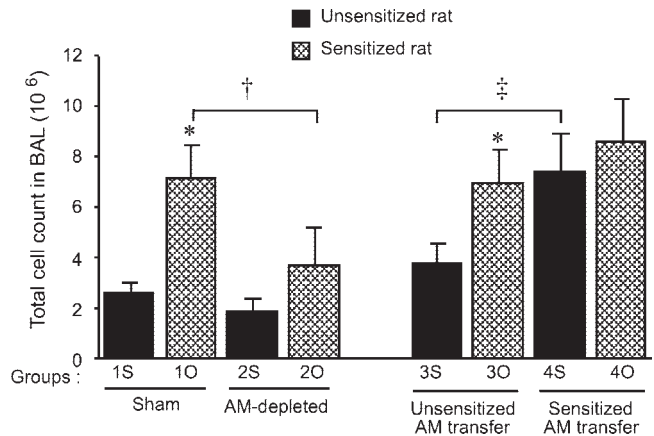


Fig. 3. Total inflammatory cell counts in BAL performed 24 h after OVA challenge. There was a significant ($P < 0.01$) increase of cell number in sensitized rats (1O and 3O) compared with unsensitized rats (1S and 3S). AM depletion significantly ($P < 0.04$) reduced the number of cells in BAL of AM-depleted sensitized rats (2O) compared with sham (1O). Transfer of sensitized AM significantly increased the number of cells present in the BAL of unsensitized animal (3S) compared with the transfer of unsensitized AM (4S). However, the transfer of unsensitized AM did not reduce the no. of cells present in the BAL of sensitized animals (3O) compared with the transfer of sensitized AM (4O). Data are means $\pm$ SE of 5 experiments. *Significant difference between unsensitized and sensitized rats. ‡Significant difference between unsensitized and sensitized AM transfer.

unsensitized rats (3S vs. 1S) or sensitized AM to AM-depleted sensitized rats (4O vs. 1O).

The protective effect of AM was also investigated in animals without AM depletion. Sensitized rats that received unsensitized AM and were challenged with saline showed an EC_{200R_L} similar (107.1 ± 11.0 mg/ml) to unsensitized animals (99.9 ± 8.0 mg/ml). However, unsensitized AM transfer reduced ovalbumin-induced airway hyperresponsiveness (83.8 ± 11.1 mg/ml) to a level not significantly different from the saline-challenged rats. Thus our data indicate that unsensitized AM can protect against airway hyperresponsiveness development.

Cells in BAL. To investigate the mechanism involved in the modulation of airway hyperresponsiveness by AM, the inflammatory process was evaluated 24 h after OVA challenge using total and differential cell counts in BAL. OVA challenge caused significant cell recruitment in sensitized (1O) rats ($7.2 \pm 1.2 \times 10^6$ cells) compared with unsensitized (1S) animals ($2.6 \pm 0.4 \times 10^6$ cells; Fig. 3). The number of cells in BAL of AM-depleted sensitized rats that received unsensitized AM (3O) was not different from AM-depleted sensitized rats that received sensitized AM (4O). However, cell counts in BAL of unsensitized animals that received sensitized AM (4S) were higher than unsensitized rats that received unsensitized AM (3S). Our data suggest that sensitized AM contribute to the inflammatory response and that unsensitized AM cannot modulate cell recruitment in sensitized rats.

There were significantly more eosinophils, neutrophils, and lymphocytes in OVA-sensitized animals (1O) than in unsensitized rats (1S) after allergen challenge (Fig. 4A). The depletion of AM decreased AM number in both sensitized and unsensitized rats without significantly affecting the number of other inflammatory cells. Transfer of unsensitized AM did not modify cell counts in unsensitized (3S; Fig. 4B) and sensitized (3O) rats compared with sham animals (1S and 1O, respectively).

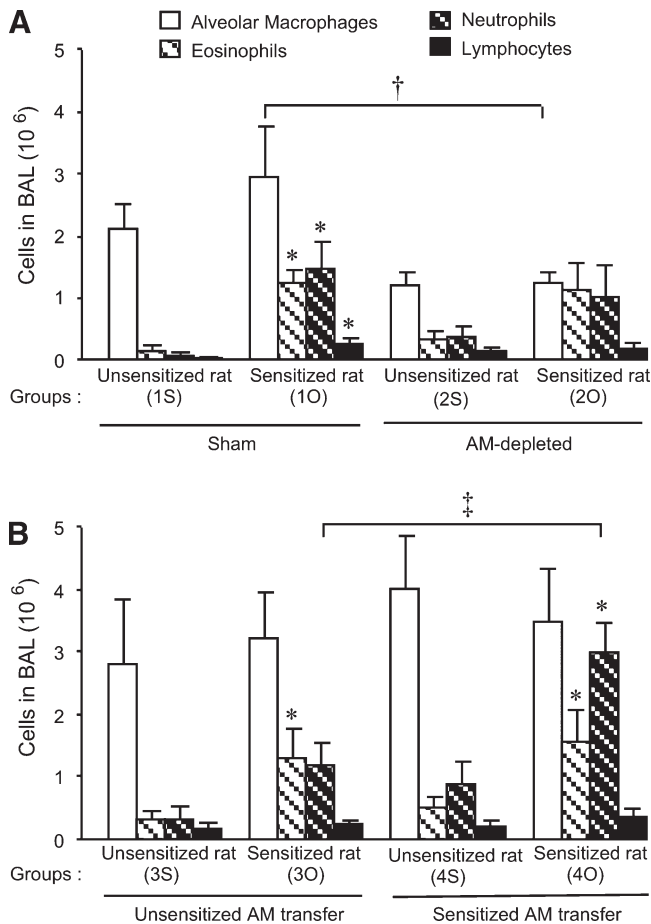


Fig. 4. Type and number of inflammatory cells in BAL performed 24 h after allergen challenge. **A**: there was a significant increase ($P < 0.04$) in eosinophils, neutrophils, and lymphocytes in sensitized animals (1O) compared with unsensitized rats (1S). The depletion of AM caused a significant reduction of AM ($P < 0.05$) in sensitized rats (2O) but not of other inflammatory cells. **B**: sensitized or unsensitized AM were transferred to sensitized or unsensitized rats 3 days after Cl₂MDP-liposome instillation. Transfer of sensitized AM significantly increased neutrophil counts in sensitized animals (4O) compared with sham (1O). Data are means $\pm$ SE of 5 experiments. *Significant difference between unsensitized and sensitized rats. †Significant difference between sham and AM depleted. ‡Significant difference between unsensitized and sensitized AM transfer.

However, transfer of sensitized AM significantly increased neutrophil number in sensitized animals (4O) compared with sham (1O). There were fewer neutrophils in BAL of sensitized animals that received unsensitized AM (3O) than in sensitized rats that received sensitized AM (4O). These data suggest that OVA-sensitized AM can promote the recruitment of granulocytes in sensitized animals, whereas unsensitized AM could not modulate the inflammatory response.

Cytokine levels in BAL fluids. To determine the role of cytokines in the modulation of airway hyperresponsiveness, levels of TNF, IL-4, IL-10, IL-12, and IL-13 were measured 24 h after allergen challenge in the first 4 ml of BAL. OVA challenge increased IL-10 release (31.2 ± 5.1 pg/ml) in BAL fluid of sensitized rats (1O) compared with unsensitized animals (1S, 13.6 ± 2.8 pg/ml; Fig. 5A). A similar, but not significant, increase in IL-10 was observed in AM-depleted rats (2O), suggesting that AM are not the major producer of IL-10 in this context or that the remaining AM produce more

IL-10. Furthermore, IL-10 levels in BAL fluid of unsensitized animals that received sensitized AM (4S) was not significantly different ($P = 0.15$) from levels in unsensitized rats that received unsensitized AM (3S). Similar data were observed with the transfer of unsensitized AM to sensitized rats. These data suggest that AM transfer does not modulate the IL-10 level.

OVA challenge increased IL-13 release (51.5 ± 25.4 pg/ml) in BAL of sensitized animals (1O) compared with unsensitized animals (1S, 2.2 ± 1.5 pg/ml; Fig. 5B). Interestingly, the depletion of AM in sensitized rats (2O, 3.5 ± 1.2 pg/ml) resulted in a significant decrease of the IL-13 level in BAL fluid, showing the importance of AM in IL-13 production. The transfer of unsensitized AM to sensitized rats (3O) as well as transfer of sensitized AM to unsensitized animals (4S) did not modulate the IL-13 level observed in the BAL compared with control (4O and 3S, respectively). IL-4 levels were also measured but were below the detection limits of the assay.

Th1 cytokine levels were also measured in BAL fluid 24 h after OVA challenge (Fig. 6). Sensitized rats challenged with OVA (1O) released significantly more TNF (123.0 ± 31.7

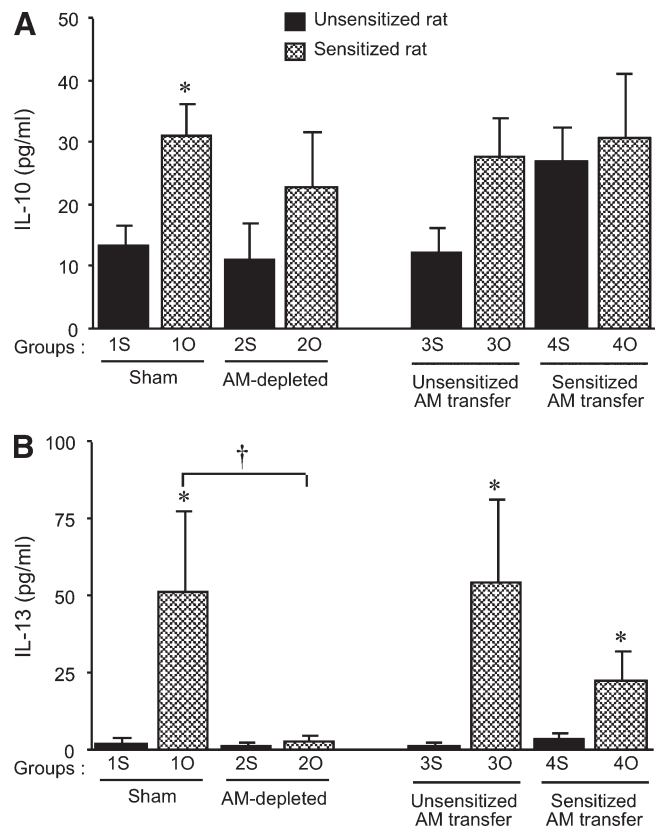


Fig. 5. Th2 cytokine (IL-10 and IL-13) levels in BAL fluids. **A**: allergen sensitization caused an increase in IL-10 level ($P < 0.03$, 1O vs. 1S), and transfer of AM did not modify it. **B**: allergen sensitization (1O) caused an increase in IL-13 level compared with unsensitized animals (1S, $P < 0.02$). Depletion of AM of sensitized rats (2O) significantly reduced IL-13 production compared with 1O. The transfer of unsensitized AM to sensitized rats (3O) and transfer of sensitized AM to unsensitized animals (4S) did not modulate IL-13 levels observed in the BAL compared with transfer of sensitized AM to sensitized rats (4O) and transfer of unsensitized AM to unsensitized animals (3S), respectively. Data are means $\pm$ SE of 6 experiments. *Significant difference between unsensitized and sensitized rats. †Significant difference between sham and AM depleted.

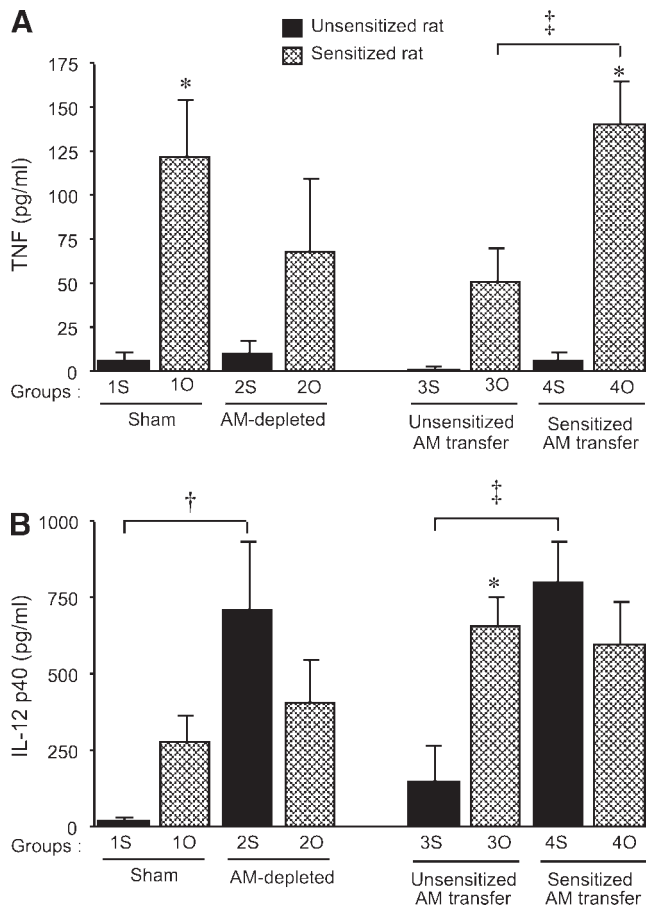


Fig. 6. Th1 cytokine (TNF and IL-12p40) levels in BAL fluids. *A*: allergen-sensitized rats (1O) released significantly more TNF ($P < 0.005$) compared with unsensitized rats (1S). TNF level in BAL fluid was significantly lower in AM-depleted sensitized rats that received unsensitized AM (3O) than in sensitized rats that received sensitized AM (4O). *B*: AM depletion of unsensitized rats (2S) caused a significant increase ($P < 0.0002$) in IL-12p40 in BAL fluid compared with control (1S). IL-12p40 level in BAL fluid of unsensitized animals that received sensitized AM (4S) was significantly higher ($P < 0.001$) than the levels of unsensitized rats that received unsensitized AM (3S). Data are means $\pm$ SE of 6 experiments. *Significant difference between unsensitized and sensitized rats. †Significant difference between sham and AM depleted. ‡Significant difference between unsensitized and sensitized AM transfer.

pg/ml; Fig. 6A) than unsensitized rats (1S, 6.0 ± 4.0 pg/ml). The depletion of AM did not significantly affect TNF release, suggesting that other cell types may contribute to the production of TNF. However, TNF level in BAL fluid was significantly lower in AM-depleted sensitized rats that received unsensitized AM (3O, 51.4 ± 18.2 pg/ml) compared with sensitized rats that received sensitized AM (4O, 141.0 ± 24.1 pg/ml). Our data suggest that sensitization of rats leads to an increase in TNF production and that unsensitized AM transfer reduced this release in BAL fluids.

OVA challenge did not significantly ($P = 0.09$) increase the release of IL-12p40 (279.9 ± 81.0 pg/ml; Fig. 6B) in sensitized rats (1O) compared with unsensitized animals (1S, 25.3 ± 6.4 pg/ml). AM depletion of unsensitized rats (2S, 711.8 ± 216.0 pg/ml) caused a significant increase in IL-12p40 in BAL fluid compared with unsensitized animals (1S). However, AM depletion did not change the IL-12p40 level in BAL of sensitized

rats (2O) compared with sham sensitized animals (1O). These data suggest that AM of unsensitized animals are important in preventing IL-12p40 overproduction in these animals. Transfer of unsensitized AM (3S), but not sensitized AM (4S), succeeded to prevent IL-12p40 overproduction observed in BAL fluid of AM-depleted unsensitized animals (2S). However, transfer of unsensitized AM to sensitized rats (3O) did not modulate IL-12p40 levels compared with the transfer of sensitized AM to sensitized rats (4O), suggesting that AM of unsensitized animals cannot prevent IL-12p40 production in sensitized animals. IL-12p70 levels were below the detection limits of the assay.

Cytokines produced by AM from BAL. To determine the role of AM in cytokine production, AM were purified from BAL 24 h after OVA challenge and cultured for 24 h. Levels of IL-10 were measured in cell-free supernatants (Fig. 7A). No

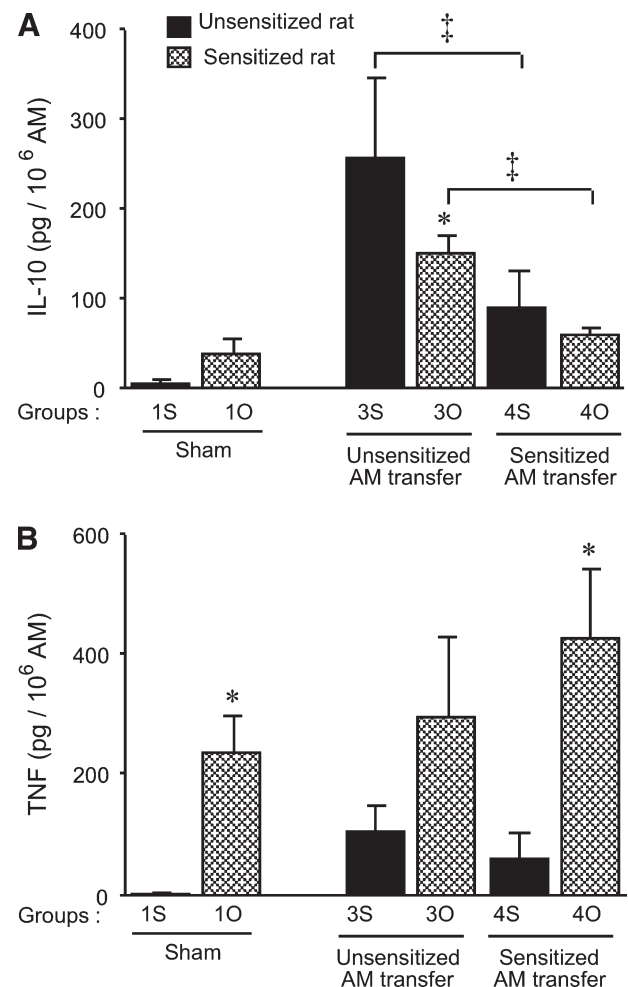


Fig. 7. Cytokines (IL-10 and TNF) produced by AM isolated from BAL. AM were purified 24 h after allergen challenge and cultured for 24 h, and cytokine levels were measured in cell-free supernatants. *A*: AM isolated from unsensitized rats that received unsensitized AM (3S) produced higher levels of IL-10 compared with AM isolated from sensitized rats that received unsensitized AM (3O). AM isolated from unsensitized rats that received sensitized AM (4S) produced lower levels of IL-10 compared with AM isolated from unsensitized rats that received unsensitized AM (3S). *B*: AM of sensitized animals produced higher levels of TNF compared with unsensitized animals ($P < 0.04$, 1O and 4O vs. 1S and 4S, respectively). Data are means $\pm$ SE of 6 rats. *Significant difference between unsensitized and sensitized rats. ‡Significant difference between unsensitized and sensitized AM transfer.



significant difference between unsensitized (1S) and sensitized (1O) AM was observed for IL-10 production. AM isolated from unsensitized rats that received unsensitized AM (3S) produced higher levels of IL-10 (256.9 ± 87.3 pg/ml) compared with AM isolated from sensitized rats that received unsensitized AM (3O, 150.2 ± 19.6 pg/ml). Interestingly, AM isolated from unsensitized rats that received sensitized AM (4S) produced lower levels of IL-10 (92.0 ± 36.8 pg/ml) compared with AM isolated from unsensitized rats that received unsensitized AM (3S), and the same results were obtained between 3O and 4O. Together, these data suggest that unsensitized AM may be important in the production of IL-10 present in BAL fluid.

Levels of TNF were also measured in cell-free supernatants (Fig. 7B). AM recovered from BAL of OVA-sensitized animals (1O) produced higher levels of TNF (237.4 ± 60.4 pg/ml) compared with unsensitized animals (1S, 0.7 ± 0.7 pg/ml). AM recovered from sensitized rats that received unsensitized AM (3O) did not produce different TNF levels compared with AM recovered from sensitized animals than received sensitized AM (4O). These data suggest that the alveolar milieu created by sensitization may influence the TNF production of AM. IL-4, IL-12, and IL-13 levels were also measured but were below the detection limits of the assay.

AM phagocytosis. To further investigate the role of sensitization in modulating various AM functions, BAL was performed 21 days after sensitization but before challenge (Fig. 1), and AM phagocytosis activity was measured. Fluorescent beads were added to AM for 6 h, and the percentage of fluorescent AM was evaluated by FACS analysis. AM from sensitized rats (1O) phagocytosed significantly fewer beads ($P < 0.05$) than AM from unsensitized (1S) rats (22.2 ± 3.0 and $40.2 \pm 5.2\%$, respectively, Fig. 8). The transfer of unsensitized AM (3O) significantly increased AM phagocytosis of sensitized rats compared with sensitized AM transfer (4O), suggesting that transfer did not modify AM phagocytosis

capacity of unsensitized AM. These results show that OVA sensitization can modulate AM functions.

DISCUSSION

We have previously demonstrated that Brown Norway rats, a widely used asthma model, develop airway inflammation and hyperresponsiveness after allergen sensitization and challenge in contrast to allergy-resistant Sprague Dawley rats (7, 8). The transfer of AM from Sprague Dawley rats to AM-depleted Brown Norway rats abrogated both the early phase reaction and airway hyperresponsiveness development, suggesting an important role for these cells in preventing asthma symptoms. We have also demonstrated that OVA sensitization without challenge differentially influences AM cytokine production (TNF, IL-10, IL-12, and IL-13) in Brown Norway and Sprague Dawley rats (8), suggesting that OVA sensitization might differently modulate AM functions in both strains of rats. In the present study, we characterized airway responsiveness and inflammation as well as AM cytokine production and phagocytosis in sensitized and unsensitized Brown Norway rats after OVA challenge. To our knowledge, this is the first study showing that unsensitized AM can protect against airway hyperresponsiveness development in sensitized allergic rats.

Airway hyperresponsiveness to a wide variety of nonspecific stimuli is a characteristic of asthma (5) and is closely related to the severity and frequency of asthma symptoms (25). To better understand the role of AM sensitization in airway hyperresponsiveness, these cells were depleted. The lack of AM per se does not cause airway hyperresponsiveness if the animal has not been previously sensitized (2S, Fig. 2), suggesting that antigen sensitization is primordial for the development of airway hyperresponsiveness to allergen challenge. This result was strengthened by the observation that transfer of sensitized AM to AM-depleted unsensitized rats (4S) did not lead to airway hyperresponsiveness. In contrast, transfer of unsensitized AM to AM-depleted sensitized rats (3O) abrogated airway hyperresponsiveness of the latter. Thus normal AM can protect sensitized animals against airway hyperresponsiveness development, but sensitized AM are unable to transfer allergy susceptibility, a phenomenon that has been attributed to T cells in a murine model (34). These data support our previous study demonstrating the protective effect of AM obtained from allergy-resistant rats and the incapacity of AM from allergic rats to transfer airway hyperresponsiveness (7). Together, our data suggest that something occurs during sensitization that eliminates the protective role of AM.

Given that airway inflammation is a feature of asthma, we characterized the inflammatory cells and cytokines in BAL after AM depletion and transfer. AM depletion significantly reduced AM number without changing the number of other inflammatory cells because of the minimal uptake of liposomes (in which CL₂MDP was given) by neutrophils and T cells (39). The number of inflammatory cells did not change when unsensitized AM were transferred to sensitized rats (3O), suggesting that the abrogation of airway hyperresponsiveness cannot be explained by the decrease of inflammatory cells in the lung. Moreover, sensitized AM recruited cells in unsensitized animals (4S) without causing airway hyperresponsiveness, indicating that inflammation in terms of the number of

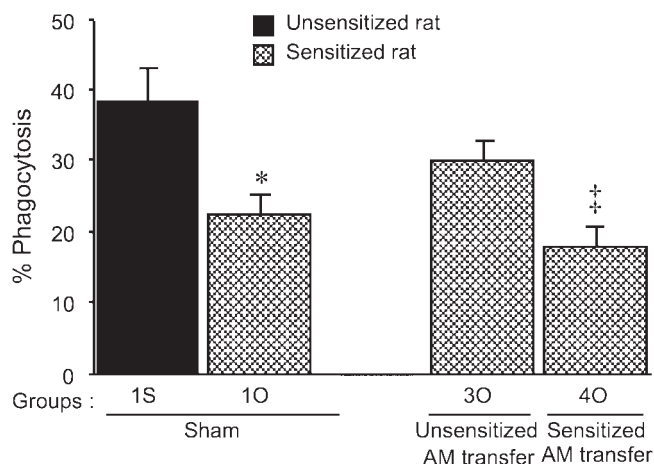


Fig. 8. AM phagocytosis after allergen sensitization. AM isolated 21 days after allergen sensitization were incubated with fluorescent beads for 6 h, and the percentage of fluorescent AM was evaluated by FACS analysis. The percentage of AM from sensitized rats that phagocytosed beads was significantly lower ($P < 0.05$) than unsensitized rats. Data are means $\pm$ SE of 6 rats. *Significant difference between unsensitized (1S) and sensitized (1O) AM. ‡Significant difference between unsensitized (3O) and sensitized (4O) AM transfer.



cells and airway hyperresponsiveness may be dissociated in our model as suggested in humans (11).

Asthma is characterized by a predominant Th2 cytokine response in the lung. To investigate the role of these cytokines in airway hyperresponsiveness, we measured levels of Th2 cytokines in BAL fluid. Transfer of unsensitized AM to sensitized rats (3O) did not reduce the increase of IL-10, suggesting that this cytokine is not involved in airway hyperresponsiveness probably because of its anti-inflammatory propriety (4). IL-10 is an important suppressive cytokine produced by a large number of immune cells, including regulatory T cells, and represents a key player in anti-inflammatory immune responses (38). Given the inflammation caused by allergen challenge, IL-10 overproduction may be seen as a way to maintain some balance. Although IL-10 has also been shown to suppress TNF production (37), it does not seem to be implicated in the modulation of airway hyperresponsiveness in our model.

IL-13 is a potent modulator of human monocyte and B cell functions (36). Our data suggest that AM are important producers of this cytokine. IL-13 was increased in sensitized rats (1O) compared with unsensitized rats (1S), but the transfer of unsensitized AM to sensitized rats (3O) did not decrease the IL-13 level, suggesting that IL-13 is not involved in airway hyperresponsiveness after allergen challenge, as shown in studies using anti-IL-13 antibody (31, 33). Thus the modulation of airway hyperresponsiveness in our model may be induced by other Th2 cytokines. Interestingly, IL-3 has been demonstrated to be important in both airway hyperresponsiveness and tissue eosinophilia (35). It is possible that unsensitized AM modulate T cell IL-3 production, but this has to be investigated further.

Given that TNF has been shown to enhance airway responsiveness in experimental animals and in human subjects (28, 46), its increased level in sensitized Brown Norway rats (1O) was not surprising. TNF induces activation of RhoA in airway smooth muscle cells that may activate intracellular signaling, contributing to airway hyperresponsiveness (23). Interestingly, the transfer of unsensitized AM to sensitized rats (3O) reduced the TNF level in BAL fluid, which may contribute to the reduction of airway hyperresponsiveness observed in these rats (Fig. 2). Furthermore, AM from unsensitized rats may down-regulate mast cell activation, known to be implicated in the quick release of TNF in BAL after allergen challenge (45), reducing the TNF level in BAL fluid.

To evaluate whether AM were directly implicated in the decrease of TNF level, AM cytokine production was assessed. The difference of TNF production between AM of unsensitized (1S; Fig. 7) and sensitized (1O) rats correlated with the level observed in BAL fluid, suggesting that AM were an important source of TNF. However, TNF production was not modulated by the transfer of unsensitized (3O) and sensitized (4O) AM to sensitized rats. These data suggest that AM modulate TNF production of other cells, but not by decreasing their own production.

IL-12, a well-characterized Th1 cytokine, can reduce airway hyperresponsiveness (27). IL-12 is a heterodimeric cytokine composed of two subunits (p35 and p40). In our model, we were unable to measure IL-12p70, but IL-12p40 was overexpressed. IL-12p40 subunit may contribute to protective Th1 immune response (10, 12) but has also been demonstrated to

form a homodimer (IL-12p80) that acts as an antagonist of IL-12 (15, 21). In our model, the depletion of AM increased the IL-12p40 level, suggesting that AM are not the only source of IL-12 in the lung, as previously reported by Walter et al. (49). Transfer of unsensitized (3O) or sensitized (4O) AM to sensitized rats did not modify IL-12p40. However, transfer of sensitized AM to unsensitized rats (4S) significantly increased IL-12p40 levels without increasing airway hyperresponsiveness, suggesting that, in our model, IL-12p40 was not related to airway hyperresponsiveness development.

To maintain lung homeostasis, AM have to perform different functions, including mediator production, cytotoxicity, and phagocytosis. To further investigate whether sensitization can modulate other AM functions, phagocytosis capacity was measured. The allergen sensitization reduced AM phagocytosis capacity in our model, as observed in asthmatic patients (3), suggesting that this altered AM function may be involved in asthma. Although the link between airway hyperresponsiveness, AM sensitization, and phagocytosis is still unknown, the reduction of AM phagocytosis activity may contribute to the perpetuation of the inflammation. Indeed, phagocytosis of dying cells by AM is an essential component in the resolution of inflammation allowing uptake of dying cells before they release their potentially toxic contents (40). Furthermore, AM that have ingested apoptotic cells develop anti-inflammatory properties (14). It is also possible that unsensitized AM phagocytose challenged OVA more efficiently than sensitized AM, preventing it from reaching other cell types that can trigger airway hyperresponsiveness.

In this study, we have focused on mediators released by AM that could influence airway hyperresponsiveness. However, cell-cell interactions that occur in sensitized rats after they have received unsensitized AM may also be important in the suppression of airway hyperresponsiveness. Costimulatory molecules like B7 (CD80 and CD86) are overexpressed on AM of asthmatic patients (1), suggesting that AM may support T cell activation. The transfer of unsensitized AM may limit the stimulation of other cell types explaining our data.

Together, our results suggest that allergen sensitization can affect AM functions. In our model, allergen sensitization was done using the following two products: aluminium hydroxide and OVA. Aluminium hydroxide alone does not affect airway responsiveness in Brown Norway rats (13). However, OVA has homology with serine protease inhibitors (44). These inhibitors are known to play an important role in asthma development in inhibiting mast cell proteases (26). The role of OVA in the modulation of AM or mast cell functions needs further investigation.

In conclusion, OVA sensitization can modulate AM functions that may be implicated in airway hyperresponsiveness development. Unsensitized AM could protect AM-depleted sensitized rats from developing airway hyperresponsiveness by reducing the TNF level in the alveolar spaces and increasing phagocytosis. The protective role of AM does not seem to be related to a reduction of inflammatory cell recruitment but may implicate other mechanisms currently under investigation. There are probably other AM mediators involved in the modulation of airway hyperresponsiveness that could act on mast cells or airway smooth muscle.



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