

Antigen Presentation by Local Macrophages Promotes Nonallergic Airway Responses in Sensitized Mice

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Local inflammatory responses involve relocating immune functions generated by previous immunization to confined parts of the body, and hence are presumed to reflect the prevailing systemic immune bias. To verify to what extent local antigen-presenting cells (APCs) may modulate immune inflammation, we analyzed the consequences of antigen presentation by macrophages on Th2-dependent airway inflammation in ovalbumin (OVA)-sensitized mice. In contrast to challenge with free OVA, which triggers airway eosinophilia and Th2 cell recruitment, intratracheal instillation of immortalized spleen macrophages (Mf4/4 cells), pulsed with OVA, promoted a nonallergic airway response featuring recruitment of interferon- γ -producing Th1 cells. Combining OVA-Mf4/4 instillation with OVA inhalation strongly reduced airway eosinophilia. Inflammation repression persisted after secondary OVA challenge and depended on the antigen-presenting ability of the macrophages. Arguing against Th1-mediated counter-regulation, Th1/Th2 ratios remained unaltered in macrophage-treated/OVA-challenged mice. In contrast, levels of interleukin-4 and interleukin-13 mRNA in lung tissue CD4⁺ T cells were strongly downregulated, indicating a suppression of Th2 cell activation. These results document a role for local macrophages/APCs in controlling the nature and intensity of local immune inflammatory responses. The resulting segregation of systemic and local levels of immune reactivity may enable local inflammation tolerance; it is a nonallergic airway response despite systemic sensitization.

Immune cells characteristically have the capacity to differentiate into subsets exhibiting differential functional and molecular properties. Besides the prominent example of Th1 and Th2 functional subsets of helper T cells (1), cytotoxic T cells (2), B cells (3), natural killer cells (4), dendritic cells (DCs) (5), and macrophages (6) also give rise to functionally different subsets, depending on the nature of the surface receptors engaged and the cytokine microenvironment. On DCs, the prominent type-1 cytokine interferon (IFN)- γ promotes mat-

uration into Th1-oriented antigen-presenting cells (APCs) (7, 8), and on macrophages induces expression of ligands involved in antigen presentation, resulting in Th1-oriented APC activity and increased cellular immune responses (9, 10). At the other end of the spectrum, alternative macrophage activation by the type-2 cytokines interleukin (IL)-4 and IL-13 or by extracellular parasites, such as helminths, induces differentiation of the activated macrophages into Th2-oriented APCs, sustaining humoral immune responses (6, 11). IL-10 and PGE₂, but also helminths and allergens, favor the development of mature DCs into type-2 effector DCs that have enhanced expression of OX40 ligand and promote the development of Th2 cells (5, 12).

Clearly, the factors governing T cell cytokine polarization are multifaceted, reflecting the functional plasticity of DCs, but also of macrophages to steer T cell activation into either Th1- or Th2-polarized effectors. Most studies on APC function focus on the role of single types of APCs, applying as experimental approaches adoptive transfer of cultured DCs (13, 14) or macrophages (9), or alternatively *in vivo* depletion of either cell type (10, 15, 16). These approaches do not necessarily take into account the opposite migration patterns of both types of APCs and their consequences for the distinct phases of immune responses. Thus, immature DCs, acting as sentinels in the peripheral tissues of the body and migrating to the draining lymph nodes after activation, will efficiently present the gathered antigen to specific naive T cells and thereby initiate the development of primary effector T cells. In contrast, macrophages, actively recruited from blood monocytes to the infected peripheral tissue, may present antigen to the infiltrating effector T cells, representing the progeny of the DC-activated naive lymph node T cells. As a consequence, tissue macrophages have the opportunity to modulate the local immune inflammatory response with respect to the systemic immunity raised by DCs. To address this issue, we adapted a mouse model for allergic asthma featuring a Th2-biased humoral immune response in the sensitization phase, and, at the local level, eosinophilic airway inflammation driven by Th2-related cytokines (17). Several studies elegantly documented the critical role of lung DCs in generating both systemic and local anti-allergen immune responses (16, 18–20). Instillation of allergen-pulsed macrophages to the respiratory tract therefore allowed determination of the extent to which the instilled macrophages interfere with the local airway inflammatory response triggered by endogenous DCs.

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Abbreviations: monoclonal antibody, mAb; antigen-presenting cells, APCs; bronchoalveolar lavage, BAL; carboxyfluorescein diacetate succinimidyl ester, CFSE; dendritic cells, DCs; enzyme-linked immunosorbent assay, ELISA; hemagglutinin, HA; hydroxymethylbilane synthase, HMBS; interferon- γ , IFN- γ ; interleukin, IL; ovalbumin, OVA; phosphate-buffered saline, PBS; ribosomal protein L13a, Rpl13a; real-time quantitative polymerase chain reaction, RT-QPCR; T cell receptor, TCR; regulatory T cells, Tr.

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Materials and Methods

Animals

Female C57BL/6 mice and BALB/c mice were purchased from IFFA Credo CR Broekman (Sulzfeld, Germany) and were housed under specific pathogen-free conditions in microisolator units.

Culture and Treatment of Macrophages

Isolation and routine culture of the murine macrophage clone Mf4/4 have been described previously (9). Twenty-four hours before pulsing with antigen, cells were grown on RPMI 1640 supplemented with 1% mouse serum. Receptor-mediated endocytosis of OVA was induced in serum-free conditions by addition of 1 μ g/ml OVA (grade V; Sigma Chemical Co., St. Louis, MO), 0.5 μ g/ml polyclonal rabbit anti-OVA IgG (ICN Pharmaceuticals, Costa Mesa, CA), and 400 U/ml of murine recombinant IFN- γ . ITS (10 mg/liter insulin, 5.5 mg/liter transferrin, and 6.7 μ g/liter sodium selenite) was used as serum substitute (Life Technologies, Paisley, UK). After 16 h, free antigen was removed by extensive washing with phosphate-buffered saline (PBS)-low endotoxin (Life Technologies).

Assays for Costimulation and Antigen Presentation

Mice were immunized by intraperitoneal injection of OVA emulsified in Ribi adjuvant (Ribi Immunochem Research, Hamilton, MT). CD4⁺ T cells were isolated from the spleen by negative selection using the StemSep method (StemCell Technologies, Vancouver, BC, Canada). For assaying Mf4/4 cell costimulatory activity, 1.5×10^5 CD4⁺ T cells were cultured for 6 h with 5×10^4 Mf4/4 cells in 96-well plates in the presence of 1.25 μ g/ml soluble anti-CD3 monoclonal antibody (mAb, clone 2C11), followed by intracellular cytokine staining. For assaying Mf4/4 APC activity, 2×10^4 Mf4/4 cells were seeded in 96-well plates and pulsed with antigen for 24 h in the presence of 400 U/ml IFN- γ . Antigen-specific T cell activation was measured using intracellular cytokine staining or by assaying T cell proliferation.

Intracellular Cytokine Staining

T cell cultures (2 h) were supplemented with brefeldin A (10 μ g/ml; Sigma) for an additional 4-h culture period. Intracellular cytokine staining was performed as described previously (21) using fluorescein isothiocyanate-conjugated anti-CD4 mAb and phycoerythrin-conjugated anti-IL-4 or anti-IFN- γ mAb (PharMingen, San Diego, CA). Fluorescence was measured with a FACScalibur flow cytometer using Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Bronchoalveolar Lavage

Mice were anesthetized with avertin (2.5% wt/vol in PBS-low endotoxin). Bronchoalveolar lavage (BAL) was performed as described (22) with 3×1 ml of Ca²⁺- and Mg²⁺-free HBSS (Life Technologies), supplemented with 0.05 mM EDTA. After centrifugation of the BAL fluid, cells were counted in a hemacytometer. Differential cell counts were determined on cytospin preparations stained with May-Grünwald-Giemsa (Sigma) by classification of 200 cells on standard morphology criteria. CD4⁺ T cells were quantified by flow cytometry using fluorescein isothiocyanate-labeled anti-CD4 mAb (PharMingen).

Sensitization, Treatment, and Challenge Protocols

Mice were sensitized by a single intraperitoneal injection of 10 μ g OVA adsorbed to 1 mg Al(OH)₃ (alum). On Day 14, 1.5×10^6 OVA-Mf4/4 cells in 80 μ l PBS were administered intratracheally, followed after 24 h by exposure to seven daily OVA aerosols (1%; 30 min) using a Jet nebulizer (Vital Signs, Totowa, NJ). Control mice were treated by intratracheal instillation of 80 μ l PBS. BAL was performed 24 h after the last aerosol challenge. When short-challenge protocols were used, mice received two additional immunizations on Days 7 and 14, and were treated with antigen-pulsed Mf4/4 cells on Days 21 and 22. On Days 27 and 28, mice were challenged by intratracheal instillation of 80 μ l OVA solution (10 μ g per mouse). BAL was performed 48 h after the last challenge.

Lung Tissue CD4⁺ T Cell Isolation

Lungs were minced and incubated for 30 min at 37°C in RPMI medium containing 150 U/ml collagenase II (Sigma), 0.02 mg/ml DNase I (Roche Molecular Biochemicals, Basel, Switzerland), and 10% fetal calf serum (Life Technologies). After washing the cells, CD4⁺ T cells were isolated by the CELLection Biotin Binder Kit according to manufacturer's protocol (DynaL A.S., Oslo, Norway).

Real-Time Quantitative Polymerase Chain Reaction

RNA isolation was performed using RNeasy (Qiagen, Crawley, UK). cDNA was synthesized using a TaqMan Reverse Transcription Reagent kit (Roche Molecular Systems, Branchburg, NJ). Real-time quantitative polymerase chain reaction (RT-QPCR) was performed on an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA), using a qPCR Core Kit for Sybr Green I (Eurogentec, Seraing, Belgium). Each RT-QPCR amplification was performed in triplicate under the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. The following forward and reverse primers were used: 5'-ATGGCTCCAGTCAGACCTTCA-3' and 5'-CAGGCAGCCACACTTCTCAA-3' (murine Muc5ac); 5'-GCCAAGCGGCTGACTGA-3', and 5'-TCAGTGAA GTAAAGG-TACAAGCTACAATCT-3' (murine IFN- γ); 5'-TCAGCCATGAAATAAC TTATTGTTTGT-3' and 5'-CCTTGAGTGTAACAGGCCATTCT-3' (murine IL-13); 5'-CCATGCTTGAAGAA GAAGCTAGTGTT-3' and 5'-GACTCATTTCATGGTGCAGCTT ATC-3' (murine IL-4); 5'-GAAACTCTGCTTCGCTGCATT-3' and 5'-TGCCCATCTTTCATCATGTATG-3' (murine HMBS); 5'-CCTGCTGCTCTCAAGGTTGTT-3' and 5'-TGGTTGTCACTGCTGCTGACTT-3' (murine Rpl13a). HMBS and Rpl13a mRNA were used as reference housekeeping genes for normalization. For each sample (x), the normalization factor was calculated using the formula $\text{mean of } Ct \text{ of ref}(i) - Ct \text{ of ref}(x)$, where i represents all samples (Ct is on the threshold cycle-value shown as the mean of three different RT-QPCR reactions). The level of target mRNA, relative to the mean of both reference housekeeping genes, was calculated by raising 2 to the power of $\{40 - [Ct \text{ of target} + \text{mean of } (Ct \text{ of norm. HMBS}; Ct \text{ of norm. Rpl13a})]\}$.

In Vivo Detection of Instilled Mf4/4 Cells

Mf4/4 cells were labeled with 10 μ M of the green fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) for 30 min at 37°C, followed by extensive washing in Ca²⁺- and Mg²⁺-free PBS-low endotoxin. 1.5×10^6 OVA-Mf4/4 cells were administered intratracheally to OVA-sensitized mice and traced 5, 24, and 48 h later in the BAL fluid, lungs,

and spleen. Lungs were minced and homogenized as described above. Numbers of CFSE-positive Mf4/4 cells were measured with a FACScalibur flow cytometer using Cell Quest software.

Cytokine Production by BAL T Cells

BAL cells (10^6 cells/ml) were pooled. Triplicate cultures were stimulated with 1 μ g/ml anti-CD3 mAb (clone 2C11) and 1 μ g/ml anti-CD28 mAb (clone 37.51; PharMingen). IL-4, IL-13, and IFN- γ levels in 24-h culture supernatant were determined by cytokine-specific enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (for IFN- γ and IL-4, Endogen, Woburn, MA; for IL-13, R&D Systems, Inc., Minneapolis, MN). All ELISAs had R-square values of > 0.99 .

Statistical Analysis

Values are expressed as mean $\pm$ SEM, unless otherwise indicated. Comparison of means between different groups was performed using the Mann-Whitney *U* test. Values of $P \leq 0.05$ are considered statistically significant.

Results

Mf4/4 Macrophages Exert APC Activity

Mature macrophages are terminally differentiated cells, exhibiting a limited life span and refractory to further expansion in culture. To ensure an unlimited source of macrophages free of contaminating cell types, features essential for analyzing the function of the cells *in vivo*, macrophages isolated from the spleen of C57/Bl6 mice and immortalized by infection with VN11 retrovirus were used (23). This provides the additional advantage over freshly isolated cells of constituting functionally and phenotypically homogeneous cell populations. From the various cell lines established and exhibiting macrophage features, clone Mf4/4 is a good representative and was used throughout our experiments. As described before, the cells express markers characteristic of mature macrophages, secrete inflammatory cytokines after activation by lipopolysaccharide, and exert antigen-presenting activity after treatment with IFN- γ (9, 24). This APC function is further illustrated in Figure 1A, showing the dependence on Mf4/4 cell costimulatory function of the induction of the Th1- and Th2-related cytokines IFN- γ and IL-4 in anti-CD3-stimulated CD4 $^+$ T cells. The Mf4/4 and anti-CD3-dependent cytokine responses were comparable to those induced in control cultures of total spleen, containing endogenous APCs (data not shown). Moreover, in the absence of anti-CD3 mAb, OVA-pulsed Mf4/4 cells stimulated the production of the Th1-related cytokine IFN- γ , but not of IL-4 (Figure 1A). The antigen-presenting ability of the Mf4/4 macrophages is further demonstrated in Figure 1B, showing the antigen (hemagglutinin)-dependent proliferation of a clonal population of Th1 cells (clone T-HA).

Absence of Airway Inflammation after Challenge with OVA-Pulsed Mf4/4 Cells

Immunization with alum-bound OVA generates a Th2-biased systemic immune response, promoting after subsequent airway exposure to OVA an eosinophilic airway inflammatory response reminiscent of allergic asthma in sensitized

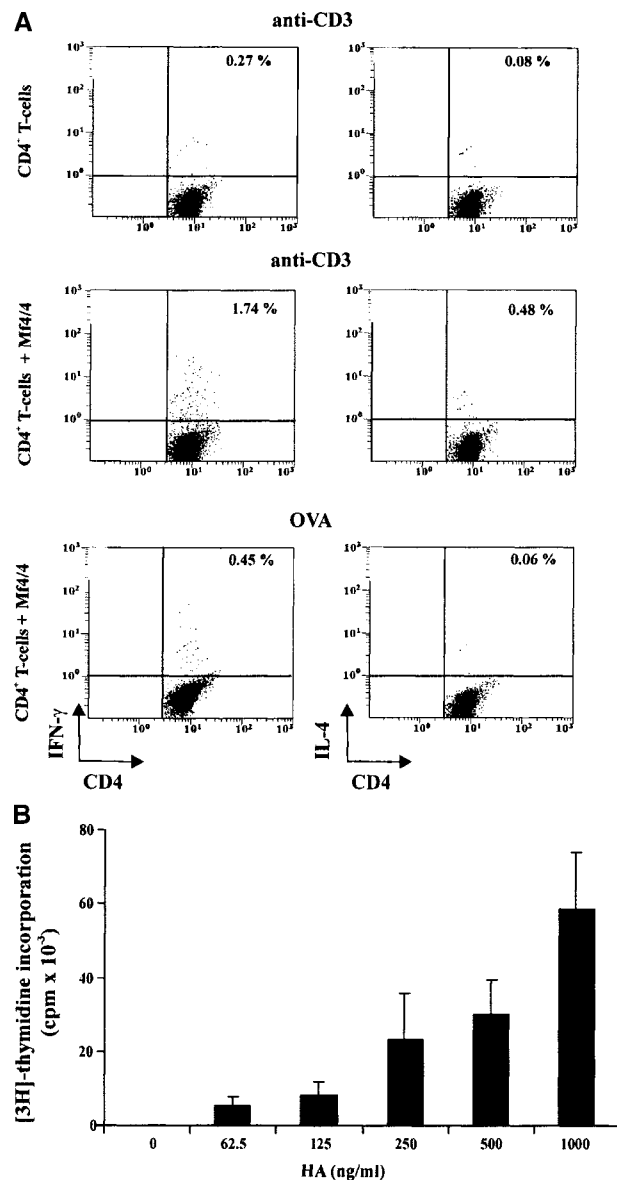


Figure 1. Mf4/4 cells exert APC activity. (A) CD4 $^+$ T cells purified from the spleen of OVA-immunized mice were co-cultured with Mf4/4 cells and stimulated with anti-CD3 mAb or specific antigen (OVA). Shown are two-parameter dot plots of CD4 and IFN- γ (left panels) or IL-4 (right panels) fluorescence intensity. Mf4/4 cells were excluded from the analysis. Percentages are double-positive cells. Background levels observed with non-pulsed Mf4/4 cells were 0.05% for either cytokine. (B) Mf4/4 cells were pulsed with increasing amounts of hemagglutinin (HA) in the presence of IFN- γ and co-cultured with HA-specific T-HA cells. T cell proliferation was assayed in triplicate cultures by [^{3} H]TdR incorporation and is expressed as mean cpm $\pm$ SD.

patients. Strikingly, introduction in the airways of OVA by intratracheal instillation of Mf4/4 cells, pulsed *ex vivo* with OVA in the presence of IFN- γ (OVA-Mf4/4), generated an uncharacteristic airway inflammation pattern, dissimilar from the pattern induced by intratracheal instillation of free antigen (Figure 2). BAL T cells, collected from sensitized mice challenged with OVA-Mf4/4 cells and stimulated *in vitro* with

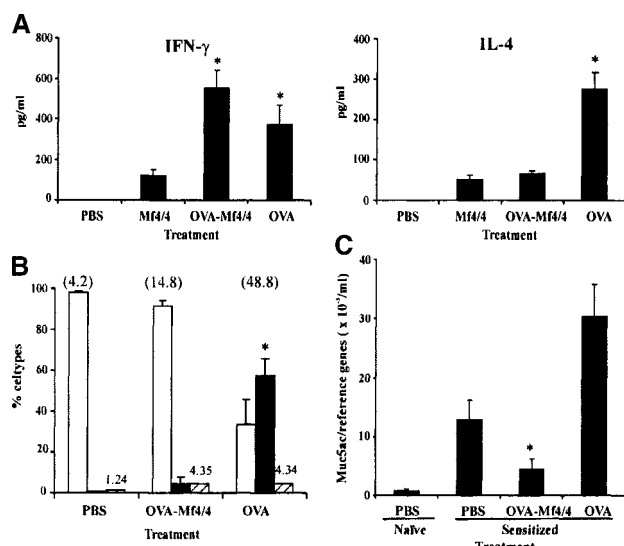


Figure 2. Absence of airway inflammation after OVA-Mf4/4 airway challenge. Sensitized mice received two intratracheal instillations of OVA-Mf4/4 cells or free OVA. Control mice received non-pulsed Mf4/4 cells or PBS as placebo. (A) T cells isolated from the BAL fluid were stimulated with anti-CD3 and anti-CD28 mAb. IFN- γ and IL-4 levels were measured in 24-h culture supernatants by ELISA. Results express the mean of triplicate cultures $\pm$ SD (* P < 0.05 versus Mf4/4-treated mice). (B) The airway inflammatory response was determined by differential cell counts of BAL cells. Percentage means of each infiltrating cell population are shown $\pm$ SEM (n = 5; * P < 0.05 versus OVA-Mf4/4-treated mice). The frequency of CD4⁺ T cells was determined by flow cytometry. Total BAL cell count ($\times 10^4$ cells/ml) is indicated between brackets. White bars, macrophages; black bars, eosinophils; hatched bars, CD4⁺ T cells. (C) Muc5ac mRNA levels in lungs were determined by RT-QPCR. Shown are means of relative Muc5ac mRNA levels, normalized against reference housekeeping genes $\pm$ SEM (n = 5; * P < 0.05 versus other groups).

soluble anti-CD3 and anti-CD28 mAb, produced high levels of IFN- γ , but only low levels of IL-4 (Figure 2A). The dependence of this particular cytokine response pattern on antigen presentation by the instilled macrophages is demonstrated by the absence of high IFN- γ levels in BAL T cells from mice instilled with empty Mf4/4 cells. In contrast, instillation of free OVA, enabling its presentation by endogenous APCs, resulted in the characteristic production of IL-4, along with IFN- γ . Also, differential cell counts on BAL cells revealed a marked difference between both types of airway challenges: in free OVA-challenged mice, an increase in cellular infiltrate was observed (48.8×10^4 /ml versus 4.2×10^4 /ml in the placebo-treated group) that was mainly attributable to eosinophils (Figure 2B). In contrast, the cellular infiltrate in the lavage of OVA-Mf4/4-treated airways nearly completely lacked eosinophils, but contained similarly increased numbers of CD4⁺ T cells. Either infiltrate was devoid of neutrophils (data not shown). In addition, mucus expression, a response that accompanies pulmonary inflammation and particularly aeroallergen-triggered airway responses, was differentially activated in sensitized mice by the different allergen formats. Enumeration by RT-QPCR of *Muc5ac* expression

in lung tissue, a function exerted mainly by goblet cells lining the bronchial cavities of the lower airways, revealed a pronounced increase in response to free OVA (Figure 2C). In contrast, instillation of OVA-Mf4/4 cells marginally increased *Muc5ac* expression compared with naive mice, but reduced the expression levels compared with placebo-treated sensitized mice.

Repression by OVA-Mf4/4 Macrophages of Free OVA-Provoked Allergic Airway Inflammation

To verify the impact of the OVA-Mf4/4-triggered airway response on free OVA-induced allergic airway inflammation, both challenges were administered consecutively. Mice, sensitized with OVA-alum, first received instilled OVA-Mf4/4 cells, followed after 24 h by a daily exposure to OVA-containing aerosol for 7 d. Analysis of the BAL revealed a significant and OVA-Mf4/4 dose-dependent reduction of the cellular infiltrate (Figure 3A). This reduction was mainly attributable to a decreased eosinophil recruitment. Absolute numbers of alveolar macrophages present in the BAL fluid remained unchanged. Also, *Muc5ac* expression in lung tissue was not affected by OVA-Mf4/4 treatment (Figure 3B). Importantly, no increase in neutrophil cell counts was observed.

A slight modification of this exposure protocol, namely a time lapse of 5 d instead of 1 d between macrophage instillation and OVA inhalation, produced an analogous suppression of eosinophilic inflammation: a significant reduction in cellular infiltration in mice first treated with OVA-Mf4/4 cells compared with mice treated with free OVA or placebo; a pronounced reduction of eosinophil infiltration; stable numbers of alveolar macrophages and absence of neutrophil infiltration (Figure 4A). Upon application of the same protocol, now however using MHC-mismatched recipients (BALB/c mice; H-2^d), intratracheal instillation of OVA-Mf4/4 cells (H-2^b) failed to reduce total cellular infiltration (data not shown). This negative result excludes cross-presentation by endogenous APCs of Mf4/4 cell-derived antigenic peptides. Therefore, a direct action of the instilled, OVA-pulsed macrophages appears to be responsible for the repression of free OVA-induced airway eosinophilia.

To verify whether the presence of instilled macrophages is necessary at the time of OVA inhalation, we verified the fate of the Mf4/4 cells during the 5-d lapse between cell instillation and free OVA inhalation. Using the cell-bound label CFSE to positively identify instilled macrophages, a clearance from the lungs of Mf4/4 cells was observed within 48 h (Figure 4B), thus below the implemented 5-d lapse. Moreover, no significant numbers of Mf4/4 cells were detected in the spleen, indicating that the cells remained tissue-bound and failed to enter the circulation. Therefore, an initial administration of OVA-Mf4/4 cells is sufficient to alter the lung responsiveness to a later allergen challenge, applied after clearance of the Mf4/4 cells.

Inhibitory Activity Depends on Antigen Presentation by Mf4/4 Macrophages

To determine the mechanism of this macrophage-mediated repressive activity, we verified its dependence on *ex vivo*

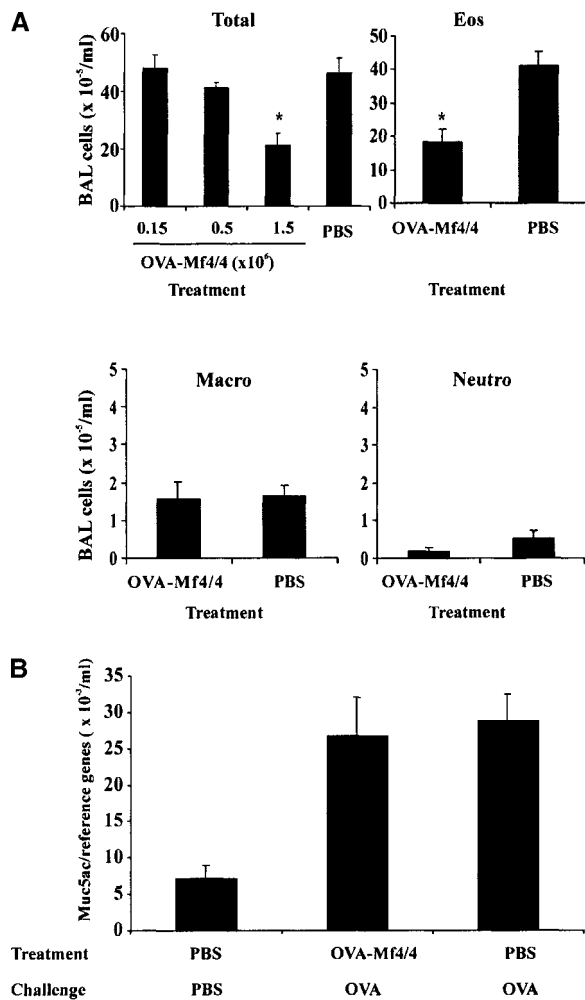


Figure 3. OVA-Mf4/4 cells repress airway inflammation induced by aerosolized OVA. (A) Sensitized mice were treated by intratracheal instillation of increasing numbers of OVA-Mf4/4 cells. Placebo control mice were treated with PBS. After 24 h, all mice were exposed on a daily basis to a series of seven OVA-aerosols. Cell counts are expressed as means $\pm$ SEM ($n = 5$; * $P < 0.05$ versus placebo-treated mice). (B) Muc5ac mRNA levels in the lungs were determined by RT-QPCR. Shown are means of Muc5ac mRNA levels normalized against reference housekeeping genes $\pm$ SEM ($n = 5$).

pulsing of the cells with OVA. Empty Mf4/4 cells, treated with IFN- γ in the absence of OVA, were instilled either 1 d or 5 d before challenge with free OVA. When applying a 1-d lapse, instillation of empty Mf4/4 cells produced a marked decrease in cell infiltration and eosinophilic inflammation (Figure 5A). In contrast, a lapse of 5 d between macrophage instillation and free OVA challenge annihilated the suppressive activity of the empty macrophages. A similar result was obtained with Mf4/4 cells pulsed *ex vivo* with the irrelevant antigen human catalase (Cat-Mf4/4) and administered 5 d before OVA challenge (Figure 5B). This differential behavior can be attributed to differences in the opportunity of the empty macrophages to spontaneously capture and present inhaled OVA. 24 h after

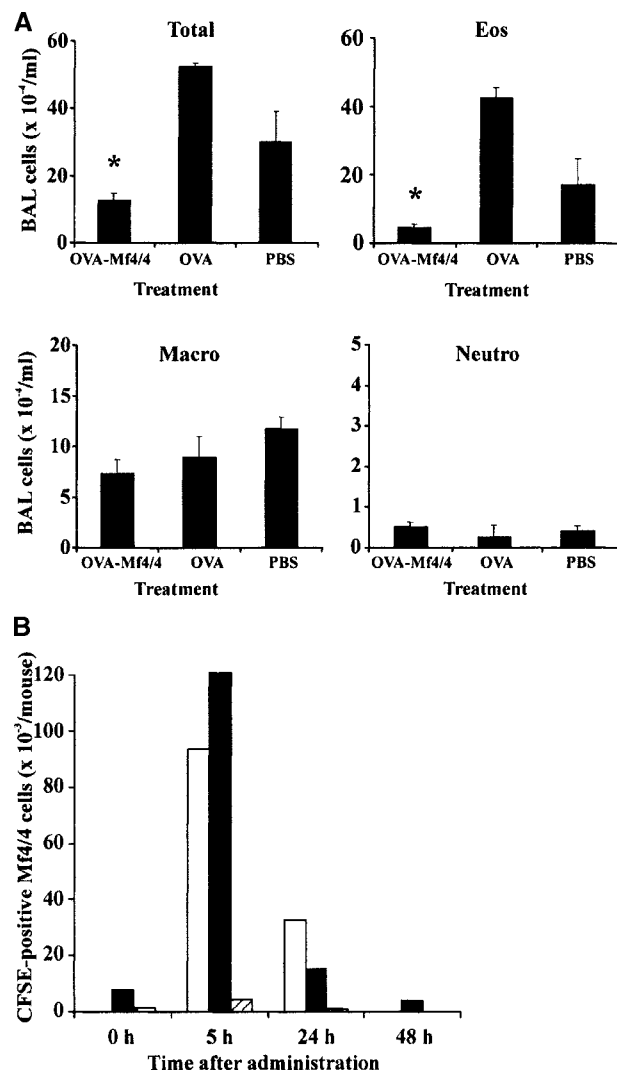


Figure 4. Persistence of airway inflammation repression. (A) OVA-sensitized mice were treated by intratracheal instillations of OVA-Mf4/4 cells, OVA or PBS. After 5 d, all mice received two additional intratracheal instillations of OVA. BAL was performed after 48 h and numbers of total cells, eosinophils, macrophages, and neutrophils present in the BAL fluid were determined. Shown are absolute numbers of the respective cell populations (mean $\pm$ SEM; $n = 5$; * $P < 0.05$ versus OVA- and placebo-treated mice). (B) Distribution of CFSE-labeled OVA-Mf4/4 cells in BAL fluid (white bars), lung tissue (black bars) and spleen (striped bars) as determined by flow cytometry (10^6 cells were analyzed for each sample). Results are expressed as the number of CFSE-positive cells present in the total organ or lavage. Three mice were analyzed per time point.

instillation, a significant number of macrophages still resided in the airways, allowing the cells to trap inhaled OVA. In contrast, after 5 d the instilled macrophages were cleared, thus abrogating their chances to capture inhaled OVA.

When assayed in culture, the capacity of Mf4/4 macrophages to present antigen to CD4⁺ T cells depends on a prior activation by IFN- γ (9). To verify whether also *in vivo* the counter-regulatory activity of Mf4/4 cells requires prior activa-

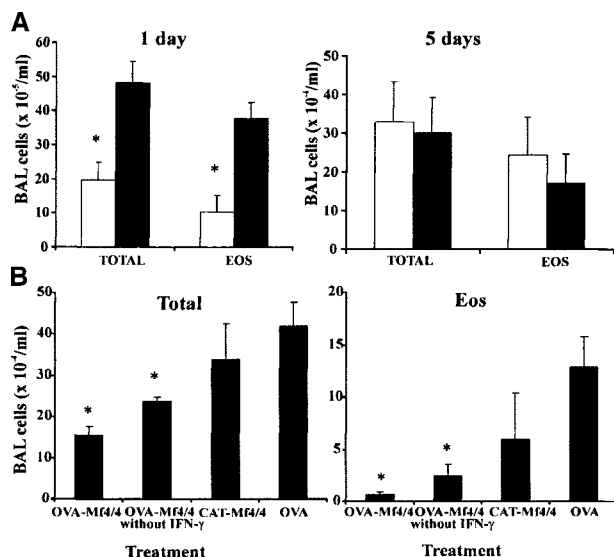


Figure 5. Repression of airway inflammation requires antigen presentation by Mf4/4 cells. (A) Sensitized mice were treated by intratracheal instillation of non-pulsed Mf4/4 cells (white bars) or PBS (black bars), followed by free OVA challenge after 1 d or 5 d. Total cell counts and eosinophil counts from BAL fluid are shown as mean $\pm$ SEM ($n = 5$; $*P < 0.05$). (B) Sensitized mice were treated by intratracheal instillation of OVA-Mf4/4 cells with or without prior activation with IFN- γ . Alternatively, Mf4/4 cells were pulsed with the irrelevant antigen human Catalase (Cat-Mf4/4). After 5 d, all mice receive two additional intratracheal instillations of OVA. Cell counts are expressed as means $\pm$ SEM ($n = 5$; $*P < 0.05$ versus OVA-challenged mice).

tion by IFN- γ , OVA-Mf4/4 cells cultured *ex vivo* with or without IFN- γ were compared. Remarkably, in both OVA-Mf4/4-treated groups reduced BAL cell counts and eosinophil numbers were observed (Figure 5B). Thus, although dependent on presentation of specific antigen, either taken up *ex vivo* or captured *in situ*, inhibition of allergen-induced airway eosinophilia by Mf4/4 macrophages is independent of prior activation by IFN- γ . We cannot, however, exclude that the instilled macrophages had been activated by endogenous IFN- γ .

T Cell Cytokine Pattern

In the absence of free allergen, OVA-pulsed Mf4/4 cells triggered a nonallergic airway response, featuring among others a Th1-cytokine pattern (Figure 2). To verify whether this particular cytokine pattern prevailed after combining OVA-Mf4/4 cell instillation with inhaled OVA challenge, BAL T cells were cultured and stimulated with anti-CD3 and anti-CD28 mAb. Strikingly, this resulted in type 1 (IFN- γ) and type 2 (IL-4 and IL-13) cytokine levels comparable to the levels detected in T cell cultures from control, placebo-treated, and OVA-challenged mice (Figure 6A). Immunofluorescent staining for BAL CD4⁺ T cells producing IFN- γ , IL-2, IL-4, or IL-5 after anti-CD3 and anti-CD28 stimulation confirmed the occurrence of similar Th cell effector profiles in both conditions (data not shown). To verify whether OVA-Mf4/4 macrophages affected instead the *in situ* activation of recruited Th cell effectors, cytokine expression

levels in lung tissue CD4⁺ T cells were determined by RT-PCR (Figure 6B). This analysis revealed a striking difference in type 1 and type 2 cytokine mRNA levels between mice exposed to combined OVA-Mf4/4 and free OVA challenges versus mice exposed only to free OVA: prior treatment by OVA-Mf4/4 cell instillation downregulated IL-4 but especially IL-13 mRNA levels while maintaining IFN- γ expression levels. Apparently, in OVA-challenged mice, macrophages/APCs do not interfere with the inflammatory mechanism of Th cell recruitment, but in contrast selectively counteract the *in situ* activation of the recruited Th2 cell effector subset.

Discussion

Hallmarks of immune defense against invading pathogens are the generation of circulating immune effector cells, thus enabling systemic immunity, and the recruitment of immune effector cells to sites of infection, thus relocating immune functions to confined parts of the body. As a consequence, regional responses are presumed to reflect the prevailing systemic immunity. However, in this equation, differential activities by local APCs interacting with recruited effector T cells and thereby controlling the local immune inflammatory response are not taken into account. Using a mouse model for allergic asthma, we verified to what extent intratracheal instillation of OVA-pulsed macrophages caused the airway inflammatory response to deviate from the prevailing, Th2-biased systemic immunity. In contrast to the pronounced eosinophilic airway inflammation caused by OVA challenge, antigen-pulsed macrophages promoted a nonallergic airway response, featuring a lack of eosinophil recruitment in the lumen of the airways and absence of *Muc5ac* expression in lung tissue. Despite the absence of inflammation, the occurrence of an airway response was apparent from the increment of T cells measured in the BAL fluid and their polarization to type-1 cytokine (IFN- γ) production. Thus, depending on the type of tissue APC engaged in presenting antigen, differential inflammatory responses are generated in mice exhibiting identical systemic sensitization. The macrophages used, namely Mf4/4 cells derived from spleen macrophages by immortalization with VN11 retrovirus, provided costimulation for both Th1 and Th2 cells, but displayed Th1-oriented APC activity when assayed *in vitro* and in naive mice (9). A similar Th1-oriented APC activity was reported for activated macrophages derived from bone-marrow progenitors (25). Both Mf4/4 macrophages and *ex vivo* macrophages therefore share a preferred induction of Th1 responses. As illustrated by our results, this function may be most pertinent locally, in inflamed tissues where the cells may counter-steer the local inflammatory response with respect to the prevailing bias of systemic immunity.

The capacity of topically administered macrophages to regulate at the local level immune inflammatory responses was further demonstrated by combining in sensitized mice OVA-Mf4/4 instillation with OVA inhalation. This yielded an $\sim 60\%$ reduction in eosinophil and total cell counts in BAL fluid compared with mice provoked only by OVA inhalation. Repression of airway eosinophilia depended on presentation of specific antigen, captured either *in situ* or

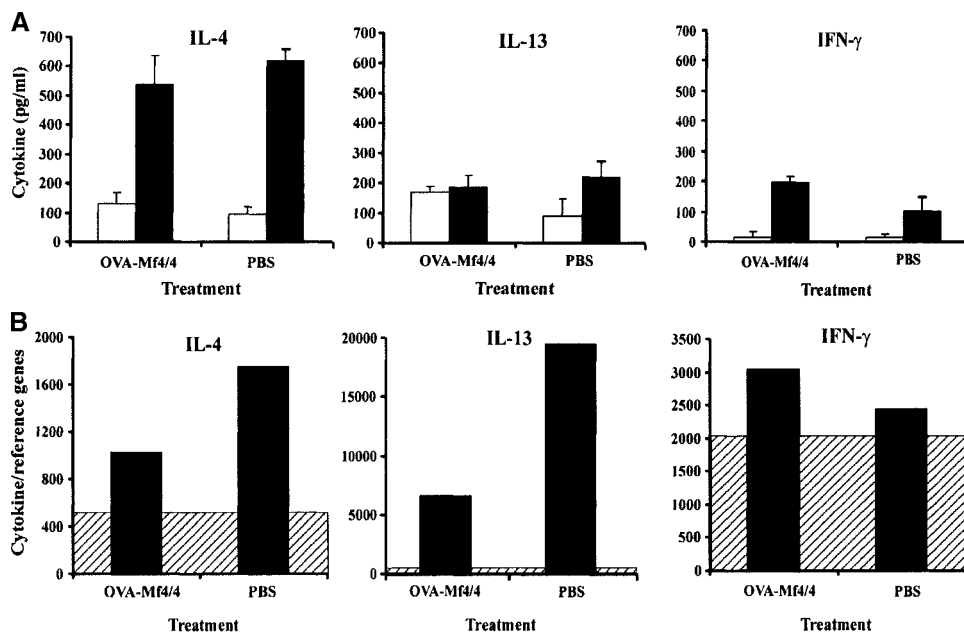


Figure 6. T cell cytokine pattern. (A) BAL cells, isolated from sensitized mice treated with OVA-Mf4/4 cells or PBS and exposed after 24 h to a series of seven OVA aerosols, were stimulated with anti-CD3 and anti-CD28 mAb (black bars). Control cultures received anti-CD28 mAb only (white bars). IL-4, IL-13, and IFN- γ levels in 24-h culture fluids were measured by ELISA. Results are expressed as mean $\pm$ SEM of triplicate cultures. (B) IL-4, IL-13, and IFN- γ mRNA levels from isolated lung tissue CD4⁺ T cells were determined by RT-QPCR. Means of relative mRNA levels, normalized against reference housekeeping genes are shown. The result is representative for two independent experiments. Cytokine expression levels of OVA-sensitized mice not challenged with OVA are indicated with a hatched square on the bars.

ex vivo. Although dependent on IFN- γ for acquiring APC activity at least in culture, stimulation of Mf4/4 cells with IFN- γ was not necessary to exert their inhibitory effects on airway eosinophilia. At present, we cannot exclude that endogenous IFN- γ acted as substitute for the *in vitro* cytokine. Compared with other tissue macrophages such as spleen macrophages from which the Mf4/4 cells originate, alveolar macrophages poorly exert APC activity (26). Nevertheless, alveolar macrophages may also repress allergic airway inflammation, as indicated by the exacerbation of allergic airway inflammation and increment of Th2-related cytokines after cell depletion by locally administered chlodronate-containing liposomes (10). Thus, similar to our observations with Mf4/4 macrophages, alveolar macrophages may initially counter-regulate the Th2-related allergic airway inflammation.

Airway exposure to allergen after clearance of the instilled macrophages still revealed a repression of the airway inflammatory response. This apparent modification of the allergic disease may result from the polarization toward type-1 cytokine (IFN- γ) production observed following instillation of OVA-pulsed Mf4/4 cells. Th1-related cytokines such as IFN- γ have been shown to inhibit IL-4 signaling (27) along with airway eosinophilia (28, 29). However, using similar mouse models for allergic airway inflammation, transfer experiments with Th1 cells failed to counterbalance allergic inflammation and on the contrary aggravated pulmonary disease (30, 31). Also, our analysis of the ratios of Th1/Th2 effector cells present in the BAL fluid did not substantiate changes in Th cell composition. Likely, additional mechanisms such as an increased induction of Tr cells contribute to the lasting repression of airway inflammation observed after intratracheal instillation of macrophages. In mouse models of allergy, several studies demonstrated repression of allergic airway inflammation by Tr cells (32,

33). Also, the pronounced decrease in IL-4 and IL-13 mRNA levels that we observed in lung tissue CD4⁺ T cells from Mf4/4-treated/OVA-challenged mice indicates a diminished activation of locally recruited Th2 cells. Notably, Shevach and coworkers showed that Tr cells feature high-affinity TCRs (34). As a consequence, weak APCs such as macrophages may promote the generation of Tr cells. Such mechanism may explain the suppression of Th2 cell activation and the lasting repression of antigen-triggered airway eosinophilia observed after instillation of antigen-pulsed macrophages.

In conclusion, our results document a role for local macrophages in controlling the nature and intensity of local immune inflammatory responses, thereby countering the systemic immune bias. Although speculative, such segregation of systemic and local levels of immune reactivity may enable local "inflammation tolerance" in immune individuals. Strikingly, inflammation tolerance has been proposed for individuals exposed to allergen and exhibiting Th2-dependent serum IgG responses, yet failing to develop allergic inflammation (35). Also, the functional features induced by locally instilled macrophages, namely diminished inflammation despite systemic immunity and local recruitment of Th effector cells, are reminiscent of inflammation tolerance.

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