Macrophage Reprogramming by Mycolic Acid Promotes a Tolerogenic Response in Experimental Asthma

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Rationale: **Mycolic acid (MA) constitutes a major and distinguishing cell wall biolipid from *Mycobacterium tuberculosis*. MA interferes with the lipid homeostasis of alveolar macrophages, inducing differentiation into foamy macrophages exhibiting increased proinflammatory function.**

Objectives: **We verified the interference of this altered macrophage function with inhaled antigen–triggered allergic airway inflammation and underlying Th2 lymphocyte reactivity.**

Methods: **Using ovalbumin (OVA) as model allergen, C57BL/6 or BALB/C mice were sensitized by OVA-alum immunization. Experimental asthma, triggered subsequently by repetitive nebulized OVA inhalation, was assessed, using as readout parameters eosinophilia, peribronchial inflammation, and Th2 cytokine function.**

Measurements and Main Results: **A single intratracheal treatment of sensitized mice with MA, inserted into liposomes as carriers, prevented the onset of OVA-triggered allergic airway inflammation and promoted unresponsiveness to a secondary set of allergen exposures. The development of this tolerant condition required an 8-d lapse after MA instillation, coinciding with the appearance of foamy alveolar macrophages. MA-conditioned $CD11b^+F4/80^+$ macrophages, transferred to the airways, mimicked the tolerogenic function of instilled MA; however, without the 8-d lapse requirement. Indicative of a macrophage-mediated tolerogenic antigen-presenting function, major histocompatibility complex (MHC)–mismatched donor macrophages failed to promote tolerance. Furthermore, Treg markers were strongly increased and established tolerance was lost after *in situ* depletion of $CD25^+$ Treg cells. Contrary to the interleukin-10 dependence of tolerogenic dendritic cells, IFN-γ deficiency but not interleukin-10 deficiency abrogated the tolerogenic capacity of MA-conditioned macrophages.**

Conclusions: **These results document an innate-driven *Mycobacterium tuberculosis* MA-triggered immune regulatory mechanism in control of pulmonary allergic responses by converting macrophages into IFN-γ–dependent tolerogenic antigen-presenting cells.**

Keywords: allergic airway inflammation; foamy macrophages; *Mycobacterium tuberculosis*; mycolic acid; tolerance

Asthma is a chronic inflammatory disorder of the airways driven by a Th2 cell response to harmless airborne environmental antigens in cooperation with eosinophils, basophils, and mast cells as distinctive constituents of the inflammatory infiltrate (1–3). Contrary to the allergenic nature of nonmicrobial airborne environmental antigens, pathogen-associated molecular patterns (PAMPs) or bacterial infections do not generally trigger asthma. Furthermore, population studies showed an inverse correlation between the clinical prevalence of asthma and the level of microbial exposure and incidence of certain infections such as tuberculosis (4). This inverse correlation gave rise to the hygiene hypothesis, stating that exposure early in life may protect the individual from the development of asthma (5, 6), and led to a renewed interest in microbial components determining T-cell reactivity to harmless non-self antigens through interaction with the mucosal immune compartment. Thus, it was suggested that regulatory-type PAMPs from the intestinal flora increase immunoregulatory control of Th2 reactions (7, 8). By promoting the development of tolerogenic dendritic cells (DCs), commensal (intestinal) bacteria or chronic pathogens may be a persistent cause of increased Treg surveillance in the body, which, by exerting bystander suppression, helps to prevent the development of allergic reactions (9, 10). Alternatively, by acting as adjuvant, bacterial agents may promote the development of allergen-specific Treg cells. Thus, heat-killed *Listeria monocytogenes* administered in conjunction with ovalbumin (OVA) to OVA-sensitized mice inhibited or reversed airway manifestations of asthma, including eosinophilia and interleukin (IL)-4 production, by promoting the development of Foxp3-expressing Treg cells (11, 12). Treatment of allergen-sensitized mice with selective Toll-like receptor (TLR)-9 ligands provided a prominent deviation of the allergenic Th2 immune response, accompanied by a reversal of established airway eosinophilia and bronchial airway hyperreactivity (13, 14). In addition, bacterial lipoprotein I (OprI) from *Pseudomonas aeruginosa*, interacting with TLR-2 and TLR-4, efficiently inhibited the Th2-cell–mediated allergic response in sensitized mice in parallel with the improvement of eosinophilic lung inflammation (15).

By promoting the differentiation of lung DCs into immunogenic or tolerogenic antigen-presenting cells (APCs), PAMPs or other microbial components may determine the development of tolerance to airborne antigens or, to the contrary, of allergic sensitization and lung inflammation (16, 17). However, relatively little is known about the role of alveolar macrophages. Alveolar macrophages represent the most abundant immune effector cell in the alveolar spaces of noninflamed lungs and together with airway epithelial cells are the first to interact with microbial components entering the respiratory tract as well as with inhaled allergen. Although macrophage activation is mostly associated with proinflammatory functions, reflecting the secretion of inflammatory cytokines and chemokines, increasing evidence

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suggests that alveolar macrophages may exert antiinflammatory functions in the initiation and progression of asthma (18, 19). Here, we verified to what extent *in situ* interaction of alveolar macrophages with mycolic acid (MA), a major and distinguishing component of the cell envelope of *Mycobacterium tuberculosis,* interfered with allergen-induced airway manifestations in sensitized mice. MA is present in mycobacteria either covalently attached to the cell wall or noncovalently associated in the form of the glycosylated derivative trehalose dimycolate (20). Recently, we demonstrated that MA interaction with macrophages promotes a reprogramming rather than acute activation of the cells, endowing the macrophages with the capacity to produce IFN-γ and myeloperoxidase on activation, whereas the production of the antiinflammatory cytokine IL-10 was partially suppressed (21). Macrophage reprogramming was accompanied with a defective lipid metabolism, resulting in the formation of foamy macrophages. The present article documents an MA-induced conversion of macrophages into tolerogenic APCs that promote $CD25^+$ Treg responses in the lungs, resulting in a pronounced and lasting suppression of inhaled allergen–triggered eosinophilic airway inflammation (22).

METHODS

Specific pathogen–free female C57BL/6 and BALB/c mice were purchased from IFFA Credo CR Broekman (Sulzfield, Germany). IFN-γ– and IL-10–deficient mice and corresponding wild-type (WT) control animals were on BALB/c genetic background. Unless otherwise specified, C57BL/6 mice were used for all animal experiments.

MA was isolated from the cell wall of a virulent strain of *M. tuberculosis* as described by Goodrum and colleagues (23) and incorporated into liposomes as described previously (19). Samples of 100 μl of these liposome suspensions were used for intraperitoneal injection or intratracheal instillation. Additional information is provided in the online supplement.

Peritoneal cells were collected 2 d after intraperitoneal injection of the liposome preparations or phosphate-buffered saline (PBS). After washing, the cells were resuspended in endotoxin-free PBS or in complete Roswell Park Memorial Institute (RPMI)–1640 medium (Life Technologies, Breda, The Netherlands). In some experiments, macrophages were enriched by a 2-h adherence step. Mice were sensitized by three intraperitoneal injections of 10 μg OVA (grade V; Sigma) adsorbed to 1 mg alum (AlOH; Sigma-Aldrich, St. Louis, MO), administered once a week. Eight days later, mice were exposed to OVA aerosol (1% in PBS for 30 min) on 2 consecutive days. In some experiments, mice received an additional set of two aerosol challenges 5 d after the last airway challenge.

MA liposomes (100 μl), liposomes, or PBS was directly applied to the airways of sensitized mice by a single intratracheal instillation 2 or 8 d before the exposure of the mice to OVA aerosol. MA-conditioned peritoneal cells (1.5×10^5 cells in 80 μl) were administered to the airways of OVA-sensitized mice on 2 consecutive days by intratracheal instillation, each followed at a 3-h interval by exposure to nebulized OVA. As controls, peritoneal cells from liposome- or PBS-treated mice, or PBS alone, were applied. In some experiments, after a 5-d rest period after exposure to an initial set of OVA aerosols, the mice received a single application of anti-CD25 (purified PC61 monoclonal antibody [mAb]) administered systemically by intraperitoneal injection (400 μg/mouse), or locally to the airways by intratracheal instillation (100 μg/mouse). The mice were then exposed to a secondary set of OVA aerosols after another rest period of 3 d.

Mice were killed and bronchoalveolar lavage (BAL) was performed 48 h after the last OVA exposure. The total volume and cell number recovered from the BAL were recorded and the cells classified by standard morphologic criteria before calculating the absolute number of each cell type. Peribronchial inflammation of hematoxylin–eosin-stained lung sections was graded in a blinded fashion using a reproducible scoring system as described previously (24).

$CD11c^+$ cells were isolated from the BAL fluid by the CELLection Biotin Binder Kit according to manufacturer's protocol (Dynal A.S., Oslo, Norway). $CD4^+$ T cells were isolated using the same procedure but from homogenized lung samples. *See* the online supplement for additional details on materials and methods.

RNA was extracted using the RNAeasy kit (Qiagen, Crawley, United Kingdom) according to the manufacturer's instructions. cDNA was synthesized using a TaqMan Reverse Transcription Reagent kit (Roche Molecular Systems, Branchburg, NJ). Real-time quantitative polymerase chain reaction (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).

Values are expressed as mean ± SD unless otherwise indicated. The Kruskal-Wallis test was used first to ascertain that significant variance existed among the groups studied. The Mann-Whitney U test was then used to test statistical significance of the differences between two groups. A p value of less than 0.05 was considered significant.

Additional detail on materials and methods is provided in the online supplement.

RESULTS

Biphasic Airway Response to Intratracheally Instilled MA

Because of the hydrophobic nature of MA, the biolipid was incorporated into liposomes as vehicle for subsequent administration into mice. Also, this resulted in a preferential targeting of phagocytes, especially macrophages as shown before (19). As a first step, we examined the characteristics of the airway response to the MA liposomes, instilled into the airways of naive mice. Analysis of the cell content of the BAL, harvested 2 and 8 d after intratracheal instillation of MA liposomes, revealed two distinct phases of inflammatory response to MA. On Day 2, a relatively small increment in the number of neutrophils was observed (Figure 1A), whereas foamy cells, a characteristic trait induced in macrophages by MA, were barely detectable. In contrast, at Day 8, the neutrophilic inflammation was resolved as was apparent from the near normal number of total cells and neutrophils, whereas a clear increase in the number of alveolar macrophages exhibiting the MA-induced foamy cell morphotype was observed (Figure 1A). Both features of the airway response to MA were absent in airways instilled with the liposome vehicle or with PBS as placebo control.

A major characteristic of foamy cells is the accumulation of intracellular lipids. Therefore, besides analysis of macrophage morphology as parameter for the presence of MA-conditioned macrophages, we verified the lipid content of the cells by applying the neutral lipid dye Nile red. Fluorescence microscopic analysis confirmed the presence of foamy macrophages 8 d after MA instillation as illustrated by the appearance of numerous yellow-labeled lipid droplets within the cells (Figure 1B).

MA Instillation Prevents Allergen-induced Airway Inflammation

To investigate whether MA would affect the airway response to inhaled allergen, we applied MA to the airways of OVA-sensitized mice either 2 or 8 d before exposure to nebulized OVA (Figure 2A). Analysis of the airway inflammatory response revealed a cumulative MA-induced neutrophilic and OVA-induced eosinophilic inflammation in mice treated with MA 2 d before OVA challenge (Figure 2B). However, MA instillation 8 d before OVA exposure suppressed the airway response to OVA as was apparent from the significant reduction in total cells and eosinophils in the BAL together with the absence of neutrophils. Again, this response was specific for MA treatment because instillation of the liposome vehicle did not affect the bronchoalveolar cell infiltrate.

To verify the transient versus persistent nature of the MA-induced protective effect, we determined whether the observed suppression of the allergic airway response persisted beyond the

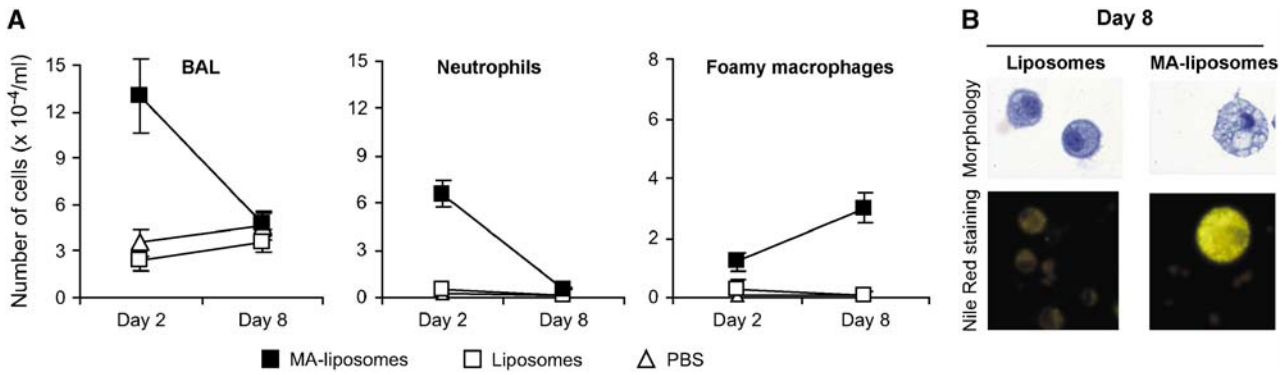

Figure 1. Airway response to mycolic acid (MA). Mice were instilled intratracheally with MA liposomes. At Days 2 and 8, the composition of the cellular infiltrate was assessed by May-Grunwald/Giemsa staining of the bronchoalveolar lavage (BAL) cells (*A*). Control mice received intratracheal injections of phosphate-buffered saline (PBS) or liposomes. Shown are the absolute numbers of total cells, neutrophils, and foamy macrophages. Data are representative of one of three separate experiments and are expressed as mean ± SEM (n = 5). (*B*) *Upper images* show the morphology of May-Grunwald/Giemsa–stained BAL macrophages, isolated 8 d after intratracheal instillation of MA liposomes or control liposomes. Lipid accumulation as a characteristic trait of foamy macrophages was confirmed by staining of the cells with the lipophylic dye, Nile red (*B*, *lower images*). The brightly labeled *yellow spots* indicate the presence of lipid droplets within the cells.

primary allergen challenges. Hence, using a long-term exposure protocol, OVA-sensitized mice treated with MA 8 d before a first set of airway allergen challenges were exposed to a secondary set of allergen challenges after a 5-d rest period (Figure 2C). Analysis of the bronchoalveolar cell infiltrate revealed a striking, near-complete suppression of eosinophilic airway inflammation (Figure 2D). In addition, inflammation within the lung tissue was suppressed as was apparent from the significant decrease in the degree of peribronchial and perivascular cell infiltration in MA-treated lungs compared with placebo or liposome-treated lungs (Figures 2E and 2F).

Decreased Th2 Cytokine Function in MA-treated Lungs

The Th2 cytokine environment, such as that induced during allergic airway inflammation, is reflected by the local macrophages acquiring a distinct functional status. Thus, polarized type 2 immune responses feature alternatively activated macrophages that express characteristic IL-4– and IL-13–dependent markers, such as arginase and Fizz-1 (25–29). As a functional readout of the local cytokine environment, we investigated to what extent the MA-triggered inflammation suppression was reflected in the expression levels of markers for alternatively activated macrophages. $CD11c^+$ cells from the BAL, containing a majority of alveolar macrophages together with some DCs (30), were isolated by positive selection after application of the long-term exposure protocol described above (Figure 2C). Real-time PCR analysis confirmed the type 2 cytokine–characteristic expression of arginase and Fizz-1, showing marked increases in expression levels in allergen-exposed, placebo-treated, or liposome-treated mice, compared with the levels observed in naive animals (Figure 3). In contrast, MA treatment prevented the OVA-induced increment in Fizz-1 and arginase expression levels.

The decreased alternative activation of the $CD11c^+$ cell subset in MA-treated airways was further confirmed when analyzing the chemokine/cytokine expression profile of the cells. In conditions where airway eosinophilia was provoked by repeated allergen exposures, $CD11c^+$ cells showed elevated mRNA levels for the eosinophil-attracting chemokines CCL8, CCL11 (eotaxin 1), and CCL24 (eotaxin 2) compared with unchallenged mice. In agreement with the suppression of eosinophilia, MA treatment prevented the expression of these chemokines (Figure 3). When assaying macrophage-derived cytokines, exposure to nebulized OVA suppressed the levels of the proinflammatory cytokine IL-18 below basal levels, whereas IL-12p40 levels remained unchanged. In turn, pretreatment with MA countered the allergen-induced suppression of IL-18 mRNA levels and promoted an increase in relative mRNA levels for IL-12p40 (Figure 3).

Regulatory T Cells Are Involved in Inflammation Suppression

We analyzed cytokine expression levels in $CD4^+$ T cells from homogenized lung tissue of mice sensitized, treated, and challenged according to the long-term exposure protocol. The isolated $CD4^+$ T cells were not further stimulated, and therefore represent the *in vivo* status of the lung $CD4^+$ T-cell subset. Although the inflammation suppression by instilled MA was accompanied by a reduction in $CD4^+$ T cells recovered from the lung tissue (data not shown), real-time PCR analysis was performed on normalized numbers of T cells. As shown in Figure 4A, no changes in the levels of type 2 (IL-4, IL-5, and IL-13) and type 1 (IFN-γ) cytokines from MA-treated mice were observed, compared with the placebo-treated group and especially the liposome vehicle control group.

Because the data do not support a Th1 cytokine–mediated counter-regulation of Th2 reactivity as a mechanism for inflammation suppression, the involvement of Treg cells was investigated by quantifying mRNA expression levels for Treg markers. Using the same purified $CD4^+$ T-cell populations, increased levels of the forkhead/winged helix transcription factor gene Foxp3, responsible for programming $CD4^+CD25^+$ regulatory T-cell development and function (31–34), were observed in the MA-treated group (Figure 4B). Also, expression levels of neuropilin-1 and glucocorticoid-induced tumor necrosis factor receptor family–related gene (GITR) were enhanced compared with the mock-treated PBS and liposome vehicle groups.

Consequently, we tested the functional involvement of Treg cells in the MA-induced tolerance of the airways to a secondary set of OVA exposures. $CD25^+$ Treg cells were depleted *in vivo* using the depleting anti-CD25 mAb PC61. Anti-CD25 mAb was administered 5 d after the first set of allergen challenges. An additional 3 d between the introduction of anti-CD25 mAb and exposure of the mice to the secondary set of OVA aerosols was allowed for the antibody to be cleared before the onset of the second wave of inflammatory reactions. Analysis of the BAL revealed that systemic depletion of $CD25^+$ Treg cells by intraperitoneally

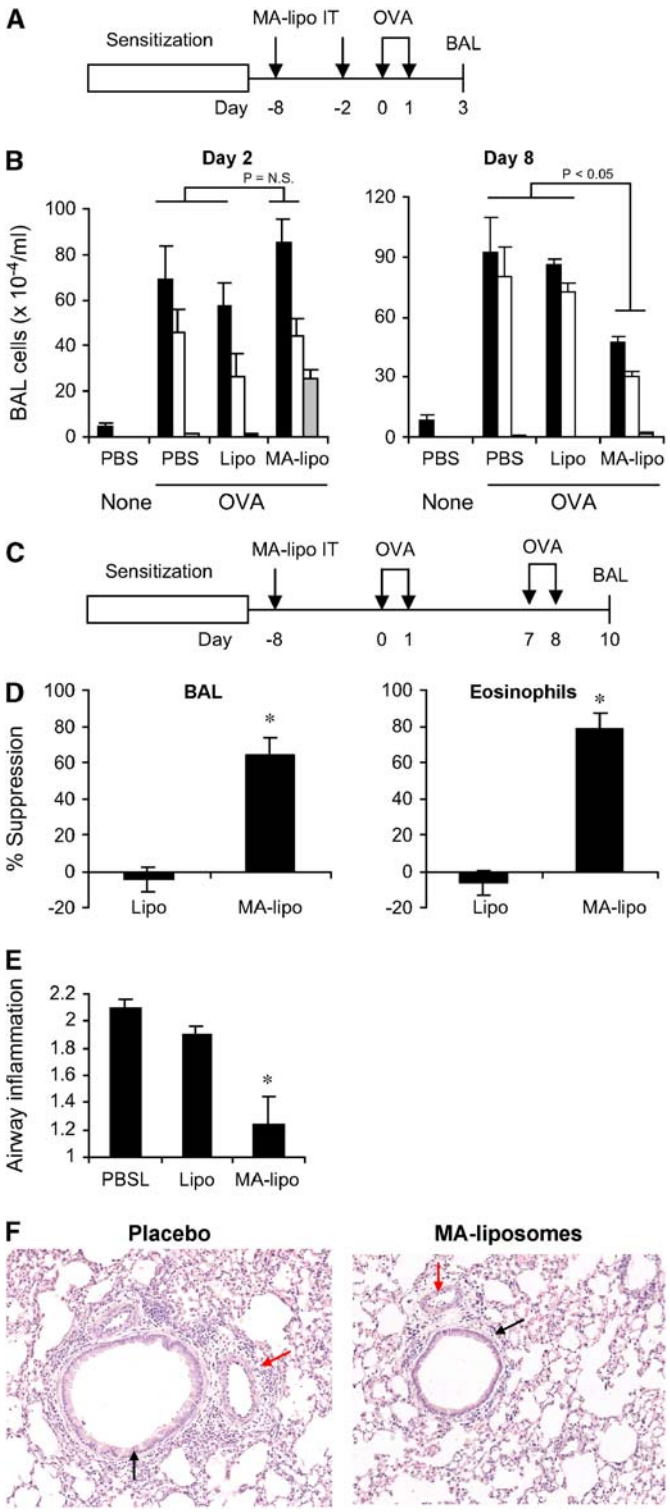

Figure 2. Intratracheal instillation of MA represses allergen-elicited airway inflammation. (*A*) Schematic diagram of the sensitization, treatment, and short-term allergen exposure protocol used. (*B*) Mice were treated as in *A* by a single intratracheal instillation of MA liposomes on Day −2 or −8. Control mice received intratracheal instillations of PBS or liposomes. All mice except a control group were exposed to aerosolized ovalbumin (OVA). Shown are the absolute numbers (mean ± SEM; n = 5) of total BAL cells (*black bars*), eosinophils (*white bars*), and neutrophils (*gray bars*). Data are representative of one of three separate experiments. (*C*) Schematic diagram of the sensitization and treatment protocol in conjunction with a long-term exposure to aerosolized OVA. (*D*) OVA-sensitized mice were treated as depicted in *C*, and the total nucleated cell counts and differential cell counts in BAL fluid were determined. Shown are the total and eosinophil cell counts indicated as percent suppression compared with placebo-treated mice (mean ± SEM; n = 5; *p < 0.05 vs. placebo-treated OVA-challenged mice). Data are representative of one of three separate experiments. (*E*) Histologic lung analysis reveals that airway instillation of MA liposomes, but not empty liposomes or PBS, decreases the inflammation around the peribronchial areas (mean ± SD; n = 5; *p < 0.05 vs. placebo-treated OVA-challenged mice). In *F*, light microscopy images of the hematoxylin–eosin-stained lung sections further illustrate the decrease in inflammatory infiltrate around the peribronchial (*black arrows*) and perivascular areas (*red arrows*) of MA-treated lungs. Lipo = liposomes.

administered mAb did not significantly affect the level of inflammation suppression in the MA-treated airways (Figure 4C). However, local depletion of $CD25^+$ Treg cells by intratracheal instillation of anti-CD25 nearly completely abrogated the MA-induced tolerance of the airways to renewed OVA challenges.

MA-reprogrammed Macrophages Mimic MA-suppressive Activity

Because MA delivery using liposomes as vehicle primarily targets macrophages (21), we verified to what extent the protective outcome of MA treatment could be mimicked by MA-conditioned macrophages. First, we assayed the total peritoneal isolate from mice injected intraperitoneally 2 d earlier with MA liposomes or, as controls, with liposome vehicle or PBS. At this time point, the peritoneal isolate contained a large fraction of foamy macrophages (data not shown). A single intratracheal instillation

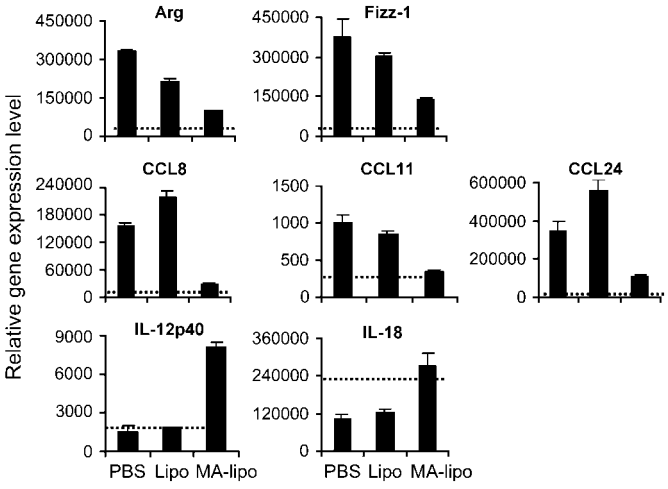

Figure 3. MA modulates the transcriptional profile of BAL $CD11c^+$ cells after allergen exposure. OVA-sensitized mice were treated intratracheally with MA liposomes, liposomes, or PBS on Day −8, followed on Day 0 by the application of the long-term OVA-exposure protocol depicted in Figure 2C. On Day 10, $CD11c^+$ cells were isolated from the BAL, and arginase (Arg), Fizz-1, CCL8, CCL11, CCL24, interleukin 12p40 (IL-12p40), and IL-18 in mRNA levels were determined by real-time quantitative polymerase chain reaction (qPCR). The *dotted line* represents values obtained from $CD11c^+$ cells isolated from naive mice. Data are expressed as relative mRNA levels, normalized against reference housekeeping genes (mean ± SD) and are representative of three separate experiments. Differences in expression levels of 2.5-fold or higher are considered significant.

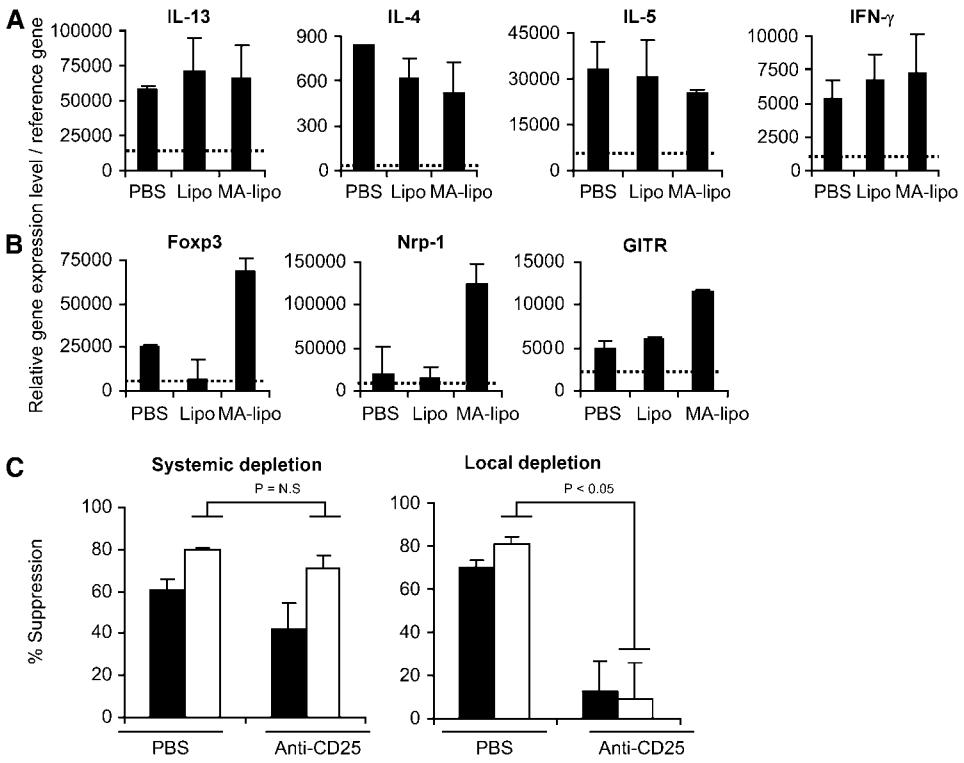

Figure 4. The T-cell partner involved in the suppressive activity of MA. OVA-sensitized mice, treated intratracheally with MA liposomes, liposomes, or PBS on Day −8 were exposed to two sets of OVA aerosols according to the long-term exposure protocol. $CD4^+$ lung T cells were isolated 48 h after the last allergen exposure (Day 10) and the relative mRNA levels of Th2 cytokines (IL-13, IL-4, IL-5) and Th1 cytokine (IFN-γ) (*A*) were analyzed by real-time qPCR, along with markers for Treg cells (Foxp3, Neuropilin-1 [Nrp-1], GITR) (*B*). Data are normalized against reference housekeeping genes and expressed as relative mRNA levels (mean ± SD). Differences in expression levels of 2.5-fold or more are considered significant. Results are representative of two separate experiments. In *C*, the functional involvement of Treg cells in the suppressive activity of MA was verified. OVA-sensitized mice were treated intratracheally with MA liposomes, liposomes, or PBS, 8 d before exposure to aerosolized OVA. After 5 d, a depleting anti-CD25 mAb was administered either systemically by intraperitoneal injection or locally by intratracheal instillation. Three days later, mice were exposed to two additional OVA aerosols before analysis of the size and composition of the inflammatory cell infiltrate. Shown are the total (*black bars*) and eosinophil counts (*white bars*) of MA-treated mice, indicated as percentage of suppression compared with placebo-treated mice (mean ± SEM; n = 5). Values (% suppression of eosinophilia) for liposome vehicle-treated mice were −8.0% for the condition where mice received a systemic depletion with anti-CD25 mAb and 5.1% for the mock-depletion control. Values for liposome-treated mice after local instillation of the depleting anti-CD25 mAb were −1.8 and 12% for the mock-depleted control. Controls on anti-CD25 were PBS placebo or isotype control mAb. Absolute cell counts for placebo-treated mice exposed to OVA challenge were 13.7 (± 2.8) × 10^5 eosinophils (used as a reference point). Minimum values for mice not exposed to allergen were 0.02 (± 0.01) × 10^5 eosinophils.

in sensitized mice of the respective peritoneal isolates was followed 3 h later by airway exposure to OVA, using the short- and long-term exposure protocols (Figures 5A and 5B). Strikingly, a single treatment with the MA-conditioned peritoneal isolate resulted in a marked tolerance of the airways to both primary and secondary sets of allergen exposures. Thus, a reduction in the bronchoalveolar cellular infiltrate and number of eosinophils was observed in both exposure protocols (Figures 5A and 5B). In addition, the numbers of infiltrating mononuclear cells remained stable and no recruitment of neutrophils was observed (data not shown). Protection from airway inflammation was an exclusive property of cells conditioned by MA, because instillation of peritoneal cells isolated from mice injected with empty liposomes or PBS as placebo failed to reduce the magnitude or composition of the bronchoalveolar cell infiltrate.

To identify the tolerogenic cell type in the MA-conditioned peritoneal isolate, the peritoneal isolate was fractionated into a macrophage-enriched, adherent cell fraction and a macrophage-depleted, nonadherent fraction. Verification by flow cytometry confirmed that over 96% of the adherent cells stained positive for the macrophage markers F4/80 and CD11b, and negative for NK1.1 and CD4. Light microscopic analysis of May-Grunwald/Giemsa–stained cells confirmed the absence of granulocytes (data not shown). Intratracheal instillation of both cell fractions and exposure to nebulized OVA showed suppression of the OVA-triggered inflammatory response only on instillation of the macrophage-enriched fraction (Figure 5C). Application of the macrophage-depleted fraction, to the contrary, slightly exacerbated the OVA-induced airway inflammation. This result identifies MA-conditioned macrophages as the cell type in the peritoneal isolate responsible for mimicking the suppressive activity of instilled MA liposomes.

Dependence on OVA Presentation by the MA-conditioned Macrophages

To verify if the suppressive activity of the MA-conditioned macrophages depended on *in situ* antigen presentation, we verified first whether the instilled macrophages required contact with inhaled OVA to exert inflammation suppression. Delaying the time of OVA aerosol challenge from 3 h after cell instillation to 5 d abolished the protective action of the instilled macrophages (data not shown). Because instilled macrophages are cleared from the airways within 2 to 3 d (35), this result indicates that macrophage contact with inhaled antigen may be critical for the MA macrophage–mediated protection against allergic airway inflammation. Accordingly, we directly verified the reliance on antigen presentation of the antiinflammatory macrophage function by applying major histocompatibility complex (MHC)–mismatched recipient mice. Transfer of the MA-conditioned peritoneal isolate from BALB/C mice to the airways of OVA-sensitized C57BL/6 mice failed to affect the airway inflammatory response provoked by subsequent exposure to OVA aerosol (Figure 6). Inversely, donor cells from C57BL/6 mice intratracheally instilled into sensitized BALB/C mice did not affect the allergen-induced airway response. However, when both donor cells and recipient mice were from the same MHC haplotype, the characteristic antiinflammatory activity was restored (Figure 6). This requirement for matched MHC haplotypes confirms that presentation of OVA by the instilled MA-conditioned macrophages constitutes a crucial step in the tolerogenic function of the cells.

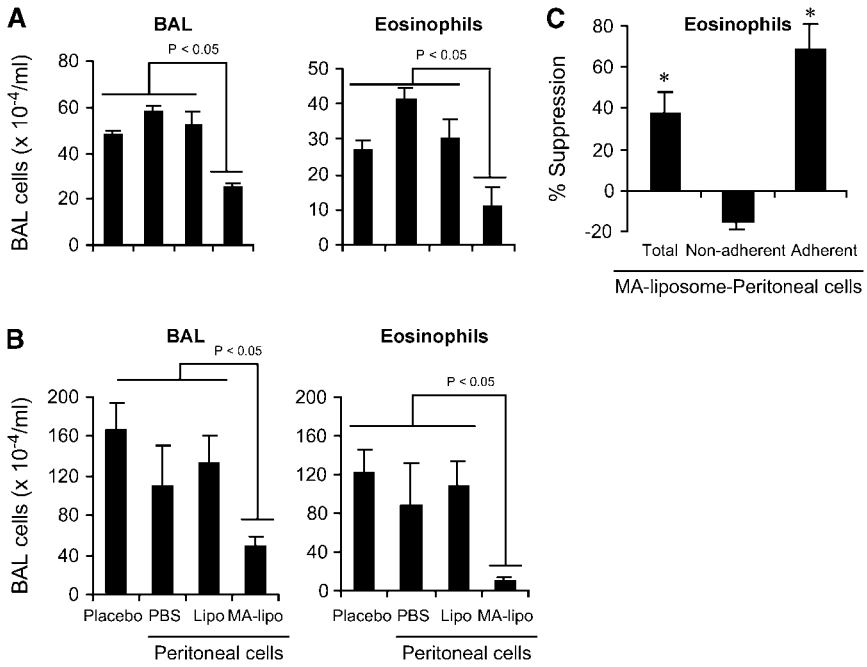

Figure 5. Transfer of MA-conditioned macrophages to the airways of OVA-sensitized mice mimics the suppressive activity of MA on allergen-induced inflammation. Peritoneal cells, isolated 2 d after intraperitoneal injection of MA liposomes, liposomes, or PBS, were instilled to the airways of OVA-sensitized mice on 2 consecutive days. Placebo controls received PBS only. Exposure to aerosolized OVA was performed 3 h after each instillation. Total nucleated cell counts and differential cell counts on BAL fluid, harvested 48 h after the last exposure, were determined. Shown are the absolute numbers of total cells and eosinophils (mean ± SEM; n = 5) (*A*). Data are representative of one of three separate experiments. *B* illustrates the persistent nature of the suppressive activity on allergen-induced inflammation. OVA-sensitized mice were treated as in *A*, but two additional OVA challenges were introduced after a 5-d rest period. BAL was performed 48 h later and the size and composition of the cellular infiltrate were determined. Shown are the absolute numbers of total cells and eosinophils of one of three separate experiments (mean ± SEM; n = 5). (*C*) MA-conditioned macrophages are responsible for the suppression of allergen-induced inflammation. OVA-sensitized mice were treated as in *A* by intratracheal instillation of either adherent or nonadherent cell fractions from MA-treated mice. The airway inflammation was quantified 48 h after the last cell instillation and OVA exposure (*B*). Eosinophil cell counts are expressed as percentage of suppression versus placebo-treated mice (mean ± SEM; n = 5; *p < 0.05).

Dependence on IFN-γ Derived from MA-conditioned Macrophages

Because the suppressive activity of MA on allergen-elicited airway inflammation could be attributed to the conditioning effect of MA on macrophages, we investigated which inherent properties enable the observed antiinflammatory function. Using the adherent, macrophage-enriched cell fraction of the peritoneal isolates from MA and mock-injected mice, analysis of the culture supernatants revealed the absence of characteristic macrophage cytokines such as IL-6, tumor necrosis factor α (TNF-α), IL-10 and IFN-γ (Figure 7A, and not shown), thus confirming the silent phenotype of the macrophages. On stimulation with LPS, all three macrophage cultures responded similarly, with a pronounced expression of IL-6, TNF-α, and IL-10 (data not shown), except for a distinct IFN-γ production specifically in MA-conditioned macrophage cultures together with a partially suppressed IL-10 production (Figure 7A). To verify whether macrophages are the cell population responsible for the observed IFN-γ production, peritoneal isolates from MA and mock-injected mice were stimulated or not with LPS and double-stained for intracellular IFN-γ and F4/80 as a macrophage-specific surface marker. Results revealed that a small percentage (8.7 ± 1.2%) of the F4/80$^+$ MA-conditioned cells stained double-positive for IFN-γ after LPS stimulation (Figure 7B). This indicates the presence of an IFN-γ–producing subset within the MA-conditioned macrophage population and may explain the relatively low IFN-γ levels detected in the culture supernatant (Figure 7A). To verify if these MA-induced features contribute to the tolerogenic function of the conditioned macrophages, MA-conditioned peritoneal isolates from IFN-γ– and IL-10–deficient mice were instilled by intratracheal route to OVA-sensitized WT mice, followed by OVA aerosol challenge. Assessment of the inflammatory response by BAL analysis revealed a failure of the IFN-γ–deficient cell instillate to affect allergen-induced inflammation (Figure 7C). In contrast, instillation of MA-conditioned IL-10–deficient cells still gave rise to a protective response, although less pronounced compared with their WT counterparts.

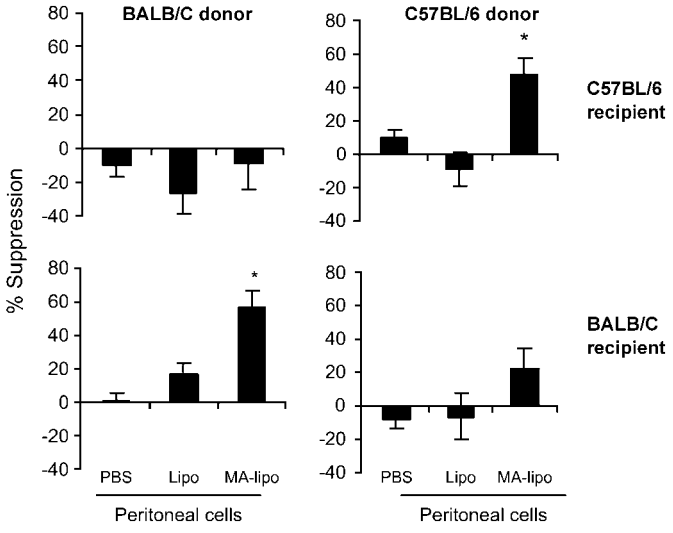

Figure 6. Suppressive activity of MA-conditioned macrophages is dependent on antigen presentation. Peritoneal cells from BALB/C and C57BL/6 mice, isolated 2 d after intraperitoneal injection of MA liposomes, liposomes, or PBS, were intratracheally instilled on 2 consecutive days to the airways of OVA-sensitized BALB/C or C57BL/6 mice (as matched and mismatched haplotypes, respectively). Each instillation was followed 3 h later by exposure to aerosolized OVA. BAL was performed 48 h after the last OVA exposure and differential cell counts were determined. The results are expressed as percentage of suppression in mice treated with MA-conditioned cells versus the placebo-treated group (mean ± SEM; n = 5; *p < 0.05).

DISCUSSION

Asthma is a prominent example of a chronic type 2 inflammatory disorder, driven by an underlying Th2 cell reactivity to inhaled

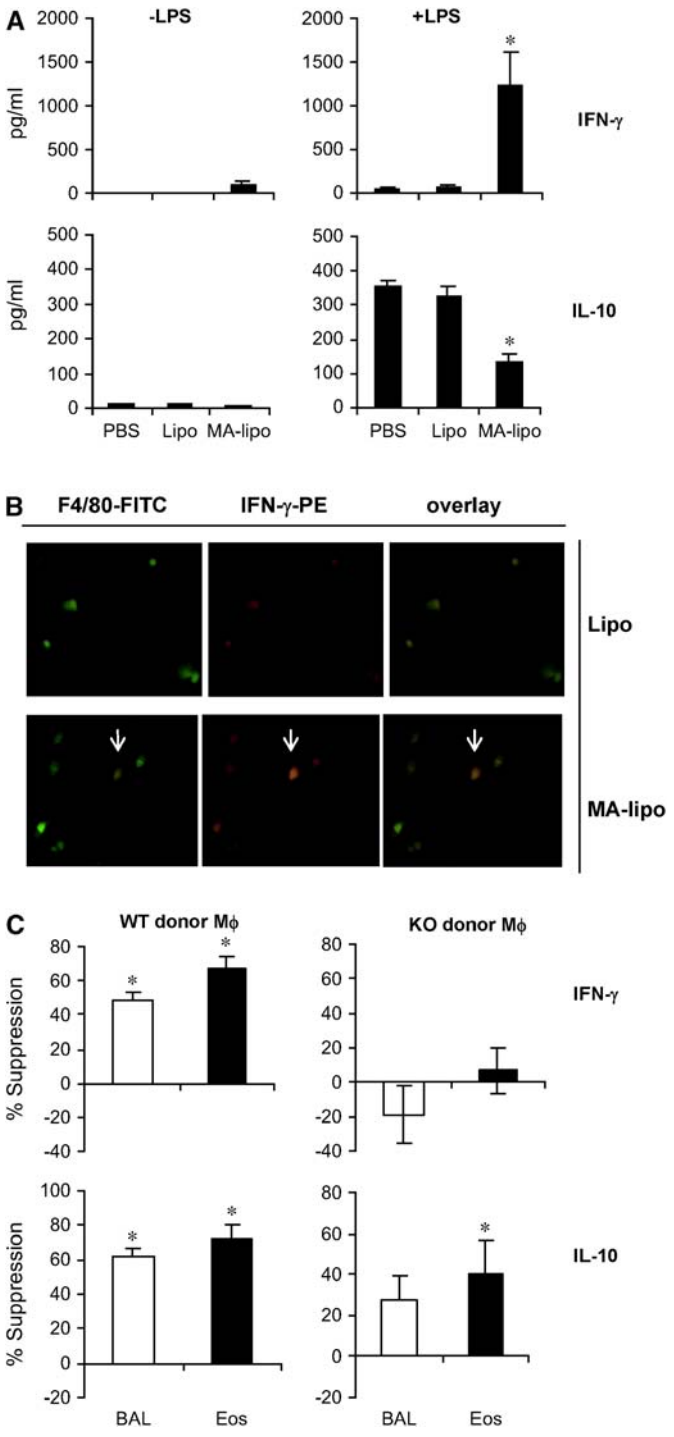

Figure 7. IFN-γ deficiency but not IL-10 deficiency abolishes the suppressive function of MA-conditioned macrophages. (*A*) Peritoneal cells were harvested 2 d after intraperitoneal injection of MA liposomes, liposomes, or PBS. After removing nonadherent cells, the macrophages were cultured as such or were additionally stimulated with LPS. Shown are the IL-10 and IFN-γ levels in the culture supernatants as determined by ELISA (mean ± SD; n = 5; *$p < 0.05$). (*B*) Alternatively, the adherent peritoneal cells were stimulated with or without LPS in the presence of brefeldin A, an inhibitor of intracellular protein transport. After fixation and permeabilization, the cells were double-stained for the macrophage-specific marker F4/80 (*green*, *first column*) and intracellular IFN-γ (*red*, *second column*) and analyzed by fluorescent microscopy. Shown are representative images of MA-conditioned and liposome control cells stimulated with LPS. Only MA-conditioned F4/80-positive cells stained positive for intracellular IFN-γ (indicated by the *white arrows*). The *last column* represents a *green* and *red* overlay image. (*C*) MA-conditioned peritoneal cell preparations obtained from IFN-γ– or IL-10–deficient mice or from the respective wild-type (WT) control mice were instilled intratracheally into OVA-sensitized mice. Peritoneal cells obtained from liposome- or PBS-treated mice were used as controls. Placebo control mice were treated with PBS only. All mice received two instillations of the respective preparations on consecutive days, followed 3 h later by exposure to aerosolized OVA. Total nucleated cell counts and differential cell counts in BAL fluid, harvested 48 h after the last instillation and exposure, were determined. Shown are the percentages of suppression of total (*white bars*) and eosinophil (*black bars*) cell numbers in mice that received MA-conditioned cells (mean ± SEM; n = 5; *$p < 0.05$).

environmental allergen (1, 2). Using a mouse model of asthma, we show that a single intratracheal instillation of the *M. tuberculosis* cell wall lipid, MA, in mice sensitized to the model allergen OVA prevents the development of allergic airway inflammation on exposure to a primary cycle of nebulized OVA and renders the airways tolerant to a secondary set of airway exposures. MA repressed inflammatory cell infiltration in the airway lumen and in the peribronchial and perivascular areas of the lung. Furthermore, whereas MA initially induced a rapid but transient neutrophil influx in the bronchoalveolar lumen, indicative of a type 1 inflammation, the tolerogenic function of MA became apparent only at later time points, after resolution of the neutrophilic inflammatory response. This uncoupling of neutrophilic inflammation from protection against allergen-induced eosinophilia was further emphasized by the similar protective activity exerted by intratracheal instillation of MA-conditioned peritoneal macrophages, a treatment not accompanied by neutrophilic inflammation. Thus, MA appears to exert its antiinflammatory action through a mechanism different from that of other microbial components, such as high-dose LPS and CpG. The type 1 inflammatory environment generated by both these TLR ligands has been proposed to be instrumental in the counteraction of allergic airway responses by deviating allergen-specific T-cell reactivity toward Th1 cells (13, 36).

Different lines of evidence indicate that MA-conditioned alveolar macrophages are involved in generating the tolerant airway condition. MA, inserted into liposomes and instilled to the airways, will primarily target macrophages present in the bronchoalveolar lumen. Also, the 8-d lapse between MA treatment and exposure to OVA, required for the development of the tolerogenic airway condition, coincides with the appearance of foamy alveolar macrophages, a characteristic outcome of the macrophage–MA interaction (19). More direct evidence derives from cell transfer experiments by intratracheal instillation of the peritoneal isolate or the adherent $CD11b^+F4/80^+$ macrophage cell fraction from mice treated 2 d before by intraperitoneal injection of MA. Cell transfer mimicked the short- and long-term suppressive functions otherwise exerted by instilled MA, and circumvented the requirement for an 8-d lapse between MA treatment and OVA exposure. An indirect mechanism, such as the transferred macrophages promoting the differentiation of DCs into tolerogenic APCs, is contradicted by the requirement for the transferred macrophages to express the same MHC haplotype as the recipient. Rather, this result indicates that presentation of antigen, captured *in situ*, by the instilled macrophages themselves is directly responsible for the observed tolerogenic function and identifies the MA-conditioned macrophages as tolerogenic APCs. However, although strongly enriched for

macrophages, we cannot exclude at the present moment that DCs, likely to be also present in the enriched cell populations, contributed to the tolerogenic APC function of the transferred cells. Further identification of the macrophage and possibly DC subsets involved in the MA-induced inflammation suppression constitutes a major challenge for the future.

Using the activation pattern of the alveolar $CD11c^+$ cell subset, consisting mainly of alveolar macrophages and a minor fraction of DCs (30), as readout of functional levels of the type 2 cytokines IL-4 and IL-13, distinguishing markers such as Fizz-1, arginase, and chemokines involved in the pathogenesis of asthma (CCL11, CCL24, and CCL8) (37) were strongly induced in alveolar macrophages from placebo-treated airways as a result of OVA inhalation. Strikingly, prior treatment with MA nearly completely abrogated the allergen-induced increment of these type 2 cytokine markers, thus indicating an MA treatment–induced deficit in Th2 reactivity in allergen-exposed airways. Complying with a suppressed Th2 cell response, mRNA levels for FoxP3, a transcription factor with expression mainly restricted to naturally occurring $CD25^+$ Treg cells (34), and the Treg markers GITR and neuropilin-1 (38, 39) were significantly increased in lung $CD4^+$ T cells after MA treatment and OVA exposure. The implication of an increased $CD25^+$ Treg cell activity, suppressing type 2 Th-cell responses to OVA and hence the airway inflammatory response to inhaled OVA, was further corroborated by $CD25^+$ Treg depletion experiments using a CD25-specific depleting mAb. Strikingly, depletion of circulating $CD25^+$ Treg did not interfere with the MA-induced tolerance of the airways to a secondary cycle of OVA exposures. However, when the depleting mAb was administered directly to the airways by intratracheal instillation, the suppressive function of MA treatment on secondary allergic airway inflammation was nearly completely disabled. This result points to a $CD25^+$ Treg population in the airways as the cell type responsible for the airway unresponsiveness to the secondary and likely also the primary allergen exposures following MA treatment. FoxP3 and CD25 markers are generally associated with naturally occurring Treg cells, generated in the thymus and specific for self-antigens. Inducible and antigen-specific $CD4^+$ Treg cells represent in contrast a more heterogeneous population. Although several reports have shown that induced regulatory T cells also up-regulate CD25 (40, 41), FoxP3 is absent in *in vitro*–induced Tr1 and Th3 cells. However, Stock and colleagues reported the induction *in vivo* of Th1-like regulatory T cells that express FoxP3 and protect against airway allergic inflammation and suggested that FoxP3 expression may be mostly restricted to *in vivo*–induced regulatory T cells (12). This agrees well with the increased FoxP3 mRNA levels we observed in lung $CD4^+$ T cells after MA treatment and OVA exposure. Strikingly, IFN-γ from MA-conditioned macrophages appears to be crucial in generating the tolerogenic response as indicated by the loss of antiinflammatory function on application of MA-conditioned macrophages from IFN-γ knockout mice. It remains an intriguing question whether IFN-γ may possibly be involved in generating a particular set of regulatory T cells, comparable to the FoxP3-positive Th1-like Treg cells described by Stock and colleagues (11), or, to the contrary, acts in an autocrine way by inducing in the conditioned macrophages the expression of T-cell–suppressive cytokines or cell surface ligands, such as transforming growth factor β, inducible costimulator receptor ligand (ICOSL), and PD-l ligand 2 (PDL2), previously described to be expressed by DCs with a Treg-promoting phenotype (42). The occurrence of an IFN-γ–dependent, alternative mechanism in promoting regulatory T-cell responses is further emphasized by the nearly unaffected tolerogenic capacity of MA-conditioned macrophages from IL-10–deficient mice, thus in sharp contrast with the dependence on IL-10 of tolerogenic DCs (42).

A striking feature of the macrophage response to MA is the development of a foam cell morphotype. Interestingly, this MA-induced feature is observed after 2 d in peritoneal macrophages and after 8 d in alveolar macrophages and coincides with the time point at which a tolerogenic conditioning is observed. Lipid metabolites resulting from excessive cholesterol accumulation in foam cells are known to act as natural ligands for nuclear lipid receptors such as peroxisome proliferator-activated receptor (PPARγ) and liver X receptors (LXRs) (43). Activation of these transcription factors not only promotes the restoration of lipid homeostasis but, in addition, can inhibit the ability of the cells to initiate inflammatory reactions (44). Because we were unsuccessful in separating the tolerance-promoting activities of instilled MA and MA-conditioned macrophages from the appearance of foam cells, both features may be causally related. This raises the intriguing possibility that the excessive cholesterol accumulation in response to MA treatment contributes to the reprogramming of the cells into tolerogenic APCs responsible for dampening effector T-cell responses.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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