Chapter 7

Farm Dust Directs Phenotype-Specific Macrophage Activation Which May Contribute to Protection Against Th2-Related Disorders

(Short Communication)


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Abstract

Farm exposures protect against allergen-induced Th2 activation, but induce neutrophilic lung inflammation. RAW 264.7 cells were differentiated into MHCII^{hi}, CD206^{hi} and IL10^{+} phenotypes and cultured with farm dust extract. Farm dust exposure inhibited CD206^{hi} and induced IL-10^{+} macrophages, suggesting a role for macrophages on the protective effect of farm exposures.

Introduction

Exposure to farm environments protects against allergic asthma and atopy (1-3). In mouse models, we and others have shown that exposure to farm dust extract (FDE) protects against allergic lung inflammation (4,5). However, it has also been shown in mice and humans that such exposures induce lung inflammation, mainly comprising neutrophils and macrophages (4,6-8).

Macrophages can differentiate into different functional phenotypes, depending on their cytokine microenvironment. In vitro, three distinct phenotypes are well established: M1, M2 and anti-inflammatory macrophages; although in vivo likely a continuum of macrophage polarization exists beyond this in vitro-based classification (9). According to recent literature, macrophages should be named after the markers used to distinguish them (10). MHCII^{hi} pro-inflammatory macrophages (or M1 macrophages) are induced by interferon (IFN)γ via the transcription factor interferon regulatory factor 5 (IRF5). They possess microbicidal properties and produce pro-inflammatory cytokines such as IL-12, IL-23, IL-1β and TNFα (11). Th2-type cytokines IL-4 and IL-13 induce CD206^{hi} (M2) macrophages, which are associated with wound healing processes. In mice, these macrophages are characterized by chitinase 3-like 3 (also known as YM1) production. IL-10^{+} (anti-inflammatory) macrophages are induced by corticosteroids, prostaglandin E2 (PGE2) or IL-10 and secrete IL-10 and transforming growth factor (TGF)β (12).

In asthmatics, elevated levels of M1 inducers (IFNγ, LPS and TNFα) have been found, especially in those with severe forms of the disease (13-15). Correlations between asthma severity and numbers of M2-dominant macrophages or their products were also shown in lungs and serum of asthmatic patients (16,17) and in mouse models (18). Studies on the role of anti-inflammatory macrophages are scarce, but it is speculated that these macrophages could play a role in the resolution phase of asthma due to their IL-10 production (12).

We have previously shown that exposing mice to FDE induced polarization to a MHCII^{hi}IRF5^{+} proinflammatory phenotype (19). Although many studies have investigated macrophage activation, there are only a few reports on the plasticity of already polarized macrophages (20). We therefore investigated the effects of FDE exposure on activated macrophage subsets, which could be related to the reported protection against Th2 responses.
Material and methods

Farm dust extract (FDE)
Settled dust samples (n=30) were collected from ridges in multiple cattle farms, pooled and FDE was prepared as described previously (4).

RAW cells culture
The leukemic macrophage cell line RAW 264.7 (EACC, Salisbury, UK) was cultured in DMEM (Invitrogen, Gaithersburg, USA) as described previously (19). Four hours after plating, cells were stimulated with either IFNγ (10 ng/ml; PeproTech, Rocky Hill, NJ), IL-13 (10 ng/ml; PeproTech, Rocky Hill, NJ) or PGE2 (5 μM; PeproTech, Rocky Hill, NJ), to induce MHC II⁺, CD206⁺ and IL-10⁺ phenotypes, respectively. Specific upregulation of each marker by each pretreatment was confirmed by flow cytometry (19).

After 48 hours of stimulation, the cells were washed three times and further stimulated for 48 hours with either IFNγ (10 ng/ml), IL-13 (10 ng/ml), PGE2 (5 μM), FDE at the concentrations of 1 μg/ml, 50 μg/ml and 100 μg/ml (final concentrations in the wells), or the combination of FDE (50 μg/ml) and IL-13 (10 ng/ml) in one solution.

After 48 hours of stimulation, cell cultures were then photographed to observe changes in morphology that could be related to changes in the milieu and cells were collected for flow cytometric analysis. Cells were incubated with 4 mg/ml Lidocaine, 10 mM EDTA and 10% fetal calf serum in phosphate-buffered saline (pH=7.3) for 15 minutes. After detachment by vigorous pipetting, cells were transferred to 1,5 ml tubes and washed 3 times with PBS supplemented with 2% FCS and 5 mM EDTA (PFE) before they were used for flow cytometry.

Flow cytometric analysis of RAW cells
Extracellular staining of MHCII and CD206 was performed using APC/Cy7-conjugated anti-MHC class II (Biolegend, San Diego, USA) and AlexaFluor 647-conjugated anti-CD206 (Biolegend), followed by intracellular staining for IL-10. An appropriate isotype control was used for the IL-10 staining (rat IgG2b-PE, eBiosciences). FACS staining procedures have been described previously (19).

Analysis was performed using a BD FACSArray cytometer (BD Biosciences) and data was analyzed using FlowJo software (Tree Star Inc., Ashland, USA). To evaluate the expression of markers of macrophage phenotypes, mean fluorescence intensity was analyzed.

Statistical methods
Data are presented as median with ranges of 4 distinct experiments. The nonparametric Kruskal-Wallis test was used to assess whether differences between groups existed, followed by a 2-sided Mann–Whitney U-test for post-hoc analysis. All analyses were performed using GraphPad Prism 5.0, and differences were considered statistically significant with p-values less than 0.05.
Results

Macrophage polarization into MHCI$^{hi}$, CD206$^{hi}$ and IL-10$^{+}$ phenotypes was induced by incubation for 48 hours with IFNγ, IL-13 and PGE2, respectively (pretreatment). Cells were subsequently incubated for another 48 hours with the same cytokines as during their pretreatment, and in addition FDE, IL-13+FDE or without second stimulation. In IFNγ-pretreated cells, upregulation of MHCI$^{hi}$ was still present even when cells were not exposed to IFNγ in the last 48 hours. The results were the same when cells received a second IFNγ stimulus or FDE stimulation (figure 1A).

IL-13-pretreated cells showed more plasticity. After 48 hours incubation without IL-13, cells lost their CD206 upregulation, while a second IL-13 stimulus induced an increase in CD206 expression. FDE stimulation did not induce CD206 expression. Since FDE exposure has been shown to prevent Th2 responses in mice and men (1-5), we investigated whether FDE exposure would prevent phenotypical changes induced by the Th2 cytokine IL-13 on macrophages. Interestingly, stimulation with IL-13 in combination with FDE prevented IL-13-induced upregulation of CD206 (figure 1B). Furthermore, we found that cells stimulated with IL-13 in combination with FDE were elongated, with similar morphology as IFNγ- or PGE2-stimulated cells, while IL-13-stimulated cells were round-shaped (figure 2). This demonstrates that FDE induces a change in morphology towards other phenotypes than IL-13-induced phenotype.

PGE2-pretreated cells expressed more IL-10 than unstimulated cells. This upregulation remained present when cells were not stimulated for 48 hours. Secondary stimulation with PGE2 or FDE in concentrations of 1 μg/ml and 50 μg/ml showed IL-10 expression similar to no secondary stimulation. A higher dose of FDE, however, induced higher IL-10 expression as compared to cells with no secondary stimulus (figure 1C).

![Figure 1](image)

Figure 1: Mean fluorescence intensity (MFI) of A) MHCI$^{hi}$, B) CD206$^{hi}$ and C) IL-10 of RAW cells that were not stimulated for 48 hours or stimulated with IFNγ (A), IL-13 (B), or PGE2 (C) to induce different phenotypes. After 48 hours of incubation, cells were incubated further with no secondary stimulus or stimulated with IFNγ, IL-13, PGE2, farm dust extract (FDE), or IL-13+FDE. Each bar represents the median with range of 4 independent experiments.*p<0.05 as compared to unstimulated macrophages, #p<0.05 as compared to IL-13, ¶p<0.05 as compared to no sec. stim.
Discussion

In this study we show that FDE exposure affects macrophage activation. While it blocks the effects of IL-13, preventing further CD206 upregulation, it also promotes anti-inflammatory IL-10 producing macrophages. Both effects suggest an important role of macrophages in the protection against Th2 responses previously described in mice and men.

An extensive body of literature describes that individuals that live or work on farms are protected against Th2-related diseases, such as atopy and allergic asthma (2,3,21). We and others have shown similar protection in mouse models of allergic lung disease, when mice were exposed to FDE (4,5). The mechanisms for such protection, however, remain unknown.

Macrophages are key innate immune cells in the lungs and depending on their polarized state play a critical role in lung homeostasis, in host defense against pathogens, coordination of the adaptive immune response, modulation of inflammation and tissue repair. Numbers of IL-4/IL-13-stimulated macrophages and levels of their products have been shown to be increased in lungs (16), bronchoalveolar lavage (BAL) and serum (17) of asthmatics. In a recent study, we have shown that numbers of CD206$^{hi}$YM1$^+$ macrophages strongly correlated with eosinophils in the lungs, suggesting that this macrophage phenotype contributes to disease severity (18). These macrophages can contribute to asthma by inducing eosinophil chemotaxis and promoting extracellular matrix production, which in turn add to remodeling (22,23). Our finding that FDE exposure blocks IL-13-induced polarization indicates a possible mechanism by which FDE exposure protects against asthma-related lung disease.

Additionally, high doses of FDE induced IL-10$^+$ anti-inflammatory macrophages, which could be beneficial to the resolution of asthma. IL-10 is an important mediator in the resolution of lung inflammation, inhibiting the production of various proinflammatory cytokines and inducing eosinophil apoptosis (11,24). Studies have shown lower levels of IL-10 production in alveolar macrophages from asthmatics as compared to healthy controls (25), indicating impaired activity of these macrophages in asthma. In addition, mice with house dust mite-induced allergic lung inflammation have lower numbers of IL-10$^+$ macrophages than control mice (18). Importantly, treatment of sensitized mice with IL-10-
producing macrophages prevents the development of allergic lung inflammation (26). Along these lines, inducing polarization towards IL-10+ macrophages could contribute to protection against allergic asthma. Macrophages could be involved in both effects of exposures to farm environments: contributing to nonallergic lung inflammation due to the increased number of MHCIIhiIRF5+ proinflammatory macrophages (19) and contributing to the protection against allergic asthma due to lower numbers of CD206hi macrophages and increased IL-10 expression by anti-inflammatory macrophages. Influencing the balance between the different macrophage subsets or improving a particular behavior of a phenotype could be a potential target for treating lung disorders. Taken together, our data show that FDE exposure blocks IL-13-mediated pro-asthmatic polarization and, in high doses, induces IL-10 expression by anti-inflammatory macrophages. Both effects of FDE may contribute to the described protective effect of farm exposures on the development of Th2-related disorders.

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Disclosures
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