

Distinct macrophage phenotypes in allergic and nonallergic lung inflammation

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Running head: Macrophage subsets in allergic and nonallergic lung disease

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24 Abstract

25 Chronic exposure to farm environments is a risk factor for nonallergic lung disease. In contrast to allergic
26 asthma, in which type 2 helper T cell (Th2) activation is dominant, exposure to farm dust extracts (FDE)
27 induces Th1/Th17 lung inflammation, associated with neutrophil infiltration. Macrophage influx is a
28 common feature of both types of lung inflammation: allergic and nonallergic. However, macrophage
29 functions and phenotypes may vary according to their polarized state, which is dependent on the cytokine
30 environment. In this study, we aimed to characterize and quantify the lung macrophage populations in
31 two established murine models of allergic and nonallergic lung inflammation by means of FACS and
32 immunohistochemistry. We demonstrated that, while in allergic asthma M2-dominant macrophages
33 predominated in the lungs, in nonallergic inflammation M1-dominant macrophages were more prevalent.
34 This was confirmed *in vitro* using a macrophage cell line, where FDE exerted a direct effect on
35 macrophages, inducing M1-dominant polarization. The polarization of macrophages diverged depending
36 on the exposure and inflammatory status of the tissue. Interfering with this polarization could be a target
37 for treatment of different types of lung inflammation.

38 Keywords (3-5 words): Macrophages, allergic asthma, farm dust, nonallergic asthma.

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41 Introduction

42 Exposure to farm environments can induce respiratory diseases such as nonallergic asthma, chronic
43 bronchitis and chronic obstructive respiratory disease (COPD) (5). We and others have shown that
44 exposure to farm dust extract (FDE) induces a marked nonallergic type of lung inflammation with
45 infiltration of neutrophils, macrophages, as well as T helper type 1 (Th1) and Th17 cells into mice lungs
46 (22, 25). Allergic asthma is typically a Th2-type disease characterized by the presence of Th2 cells,
47 eosinophils, macrophages and cytokines such as interleukin (IL)-4, IL-5 and IL-13 (30). Interestingly, a
48 common feature of both allergic and nonallergic asthma is the increased numbers of macrophages in the
49 lungs.

50 Lung macrophages are important innate immune cells, which play critical roles in lung homeostasis, host
51 defense against pathogens and resolution of inflammation. These diverse functions of macrophages are
52 achieved by the plasticity of these cells, which, depending on signals present in their microenvironment,
53 can polarize into a plethora of different phenotypes (14, 28). Cytokines such as Interferon (IFN) γ and
54 tumor necrosis factor (TNF) α , or bacterial products, such as lipopolysaccharide (LPS) induce polarization
55 into pro-inflammatory macrophages through the transcription factor interferon-regulatory factor 5 (IRF5).
56 These macrophages, loosely called M1-dominant, release proinflammatory cytokines IL-12, IL-1 β and
57 TNF α and are important in host defense against intracellular pathogens (9, 14).

58 Macrophages induced by the pro-allergic asthma cytokines IL-4 and IL-13, also known as M2-dominant
59 macrophages, are important in wound healing and host defense against helminth infections and are
60 characterized by upregulation of the mannose receptor (CD206) and, in mice, production of the chitinase-
61 like protein YM1. Anti-inflammatory macrophages are induced by compounds and mediators like
62 corticosteroids, IL-10 or prostaglandin 2 (PGE2). These anti-inflammatory macrophages are also
63 characterized by upregulation of CD206, but produce the anti-inflammatory cytokine IL-10 (1).

64 We and others have shown that asthmatics (12) and mice with allergic airway inflammation have higher
65 numbers of M2-dominant macrophages and their products in lung tissue (7, 11, 12, 15), serum and
66 bronchoalveolar lavage (3) as compared to controls. In addition, numbers of this macrophage phenotype

67 have been associated with disease severity both in mice and humans (4, 12) and these macrophages may
68 contribute to the development of allergic airway inflammation (6, 10, 11, 13).
69 In mice exposed to FDE, we and others noticed higher numbers of macrophages in lung tissue (19, 25).
70 However, the phenotype of these macrophages has not been investigated yet. In this study we therefore
71 compared macrophage phenotypes in allergic and nonallergic lung inflammation and used a macrophage
72 cell line to investigate the direct effect of FDE exposure on macrophage polarization.

73 **Material and methods**

74 **Farm dust extract (FDE)**

75 Settled dust samples (n=30) were collected from ridges in multiple cattle farms. Dust was pooled and FDE
76 was prepared as described previously (25). Briefly, aliquots of ~2.5 gram pooled dust were mixed with
77 12.5 gram glass beads and sterile water was added to a volume of 50 ml in a Greiner tube, mixed for 5 min
78 on an end-over-end roller at room temperature and subsequently sonicated for 5 min in an ultrasonic
79 bath with crushed ice. Afterwards, aliquots were pooled in a glass Erlenmeyer and sterile water was
80 added to a final concentration of 10 mg dust per ml, and sodium chloride solution was added till a final
81 concentration of 0.9%. Erlenmeyers were shaken for 6 hours at room temperature. Then samples were
82 centrifuged at 20.000g/4°C/45 min and after centrifugation the dust extract was dialyzed (MWCO 3500)
83 against DI-distilled water, sterilized through filtration through a 0.22 um filter and lyophilized.

84 Endotoxin concentration of FDE was assessed using the limulus amoebocyte lysate assay (Lonza, Verviers,
85 Belgium) as described previously (25). One mg/ml FDE contained 9941 endotoxin units (EU)/mg.

86 **Animals**

87 Specific pathogen free female BALB/c mice (aged 6-8 weeks, Harlan, Horst, The Netherlands) were housed
88 in groups of 6-8 mice each and had access to standard food and water *ad libitum*. An established model
89 for intranasal instillation of FDE or house dust mite (HDM, whole bodies from *Dermatophagoides*
90 *pteronyssinus*, Greer Laboratories, Lenoir, USA) was used (25). Briefly, mice were anesthetized with
91 isoflurane and intranasally exposed to FDE (1 mg/ml in PBS, 50 µl/day), HDM (40 µg/day in 50 µl PBS) or
92 sterile PBS (50 µl/day) as a control, four times per week, during six consecutive weeks. All animal
93 protocols were approved by the Institutional Animal Care and Use Committee of the University of
94 Groningen (Permit Number: 6615A) and procedures were performed under strict governmental and
95 international guidelines.

96 **Tissue collection**

97 After 6 weeks of exposure to either HDM or FDE, mice were anaesthetized and sacrificed by cardiac
98 exsanguination. From 8 mice per group, the left lung lobe was snap frozen and kept in -80°C until

preparation of lung homogenates for cytokine analysis. Right lung lobes were carefully inflated with 0.9 ml 50% Tissue Tek, O.C.T. (SaKura, Alphen aan den Rijn, The Netherlands) in PBS, and three of the four lobes were snap frozen and stored at -80°C until use, while the smallest right lung lobe was formalin-fixed and embedded in paraffin. From 6 mice per group, lungs were removed and kept in cold PBS for lung cell isolation.

Lung homogenates

Snap frozen lung tissue was mechanically homogenized (50% w/v) in 50 mM Tris-HCl buffer, containing 150 mM NaCl, 0.002% Tween-20 (pH 7.5) and a protease inhibitor (Sigma Aldrich, Zwijndrecht, The Netherlands). Homogenates were centrifuged at 12000xg for 10 minutes to remove any insoluble material. Supernatants were subsequently stored at -80°C until further analysis.

Lung cell isolation

Lungs were minced and incubated for 45 min at 37°C in RPMI medium supplemented with 10% fetal calf serum (both Lonza, Verviers, Belgium), 10 µg/ml DNase I (grade II from bovine pancreas, Roche Applied Science, Almere, Netherlands), and 0.7 mg/ml collagenase A (Sigma-Aldrich) in a shaking water bath. After that, the digested lung tissue was passed through a 70 µm nylon strainer (BD Biosciences, Breda, Netherlands) to obtain single cell suspensions. To lyse contaminating erythrocytes, cell suspensions were incubated with 10 times diluted Pharmlyse (BD Biosciences). Cells were centrifuged through 70 µm strainer caps and counted using a poch-100i hematology analyzer (Sysmex, Mundelein, USA), before they were used for flow cytometry.

Flow cytometric analysis

Single lung cell suspensions were stained for macrophage subsets using the following directly labeled antibodies: APC/Cy7-conjugated anti-CD11c (Biolegend, Fell, Germany), Percp/Cy5.5-conjugated anti-CD11b (Biolegend), PE-conjugated anti-CD206 (Biolegend), A700-conjugated anti-MHC class II (Biolegend), Pacific Blue-conjugated anti-F4/80 (Biolegend), Alexa Fluor647-conjugated anti-ICAM1 (Biolegend). Approximately 10^6 cells were incubated with the antibody mix including 1% normal mouse serum for 30 minutes on ice, protected from light. After washing the cells with PBS supplemented with 2% FCS and 5 mM EDTA (PFE), cells were incubated with FACS lysing solution (BD Biosciences) for 30 minutes on ice.

126 After that, cells were washed once with PFE, and resuspended in PFE and kept in the dark on ice until flow
127 cytometric analysis. The fluorescent staining of the cells was measured on a LSR-II flow cytometer (BD
128 Biosciences) and data were analyzed using FlowJo Software (Tree Star, Ashland, USA).

129 Cytokine analysis

130 Concentrations of cytokines were measured in lung homogenates by multiplex ELISA system (Affimetrix,
131 High Wycombe, UK). Cytokines measured: IL-1 β (lower limit of detection-LLD= 0.16 pg/ml), IL-4 (LLD= 0.11
132 pg/ml), IL-5 (LLD= 0.22 pg/ml), IL-10 (LLD= 0.75 pg/ml), IL12p70 (LLD=0.13pg/ml), IL-13 (LLD= 0.23pg/ml),
133 IL-17A (LLD=0.08 pg/ml), TNF α (LLD=0.12 pg/ml) and IFN γ (LLD- 0.1pg/ml). YM1 (LLD= 60.1 pg/ml) was
134 measured in lung homogenate by standard ELISA (R&D Systems, Oxon, UK).

135 Histology

136 Sections (3 μ m) of formalin-fixed and paraffin-embedded lung tissue were stained for different
137 macrophage subsets using a general macrophage marker, Mac3 (rat anti-Mac3, BD Biosciences), in
138 combination with phenotype-specific markers using standard immunohistochemical procedures. To
139 visualize Mac3, an immuno alkaline phosphatase procedure was used with Fast Blue BB salt (Sigma
140 Aldrich) as chromogen. Numbers of M1-dominant macrophages were determined by double staining of
141 Mac3 and IRF-5 (rabbit anti-IRF-5, Proteintech Europe, Manchester, UK) , M2-dominant macrophages
142 were determined by double staining of Mac3 and YM1 (goat anti-mECF-L, R&D Systems), and anti-
143 inflammatory macrophages were identified by double staining of Mac3 and IL-10 (rabbit anti-IL-10, Hylcult
144 Biotech, Uden, The Netherlands). IRF-5, YM1 and IL-10 were visualized with 3-amino-9-ethylcarbazole
145 (AEC, Sigma Aldrich) as chromogen. Double-positive cells were counted manually in parenchymal lung
146 tissue and numbers were corrected for the total area of lung tissue section as assessed by morphometric
147 analysis using Aperio ImageScope viewing software 11.2.0.780 (Aperio, Vista, USA).

148 RAW cells culture

149 RAW 264.7 macrophages (EACC, Salisbury, UK) were cultured in DMEM (Invitrogen, Gaithersburg, USA)
150 enriched with 10% fetal calf serum, 10 μ g/ml Gentamycin (Invitrogen,) and 2mM L-Glutamine (Invitrogen).
151 Cultures were split 1:4-1:8 twice a week, with careful scraping in their old medium. Then, they were
152 transferred to a new T75 culturing flask (Corning, New York, USA), with new medium.

153 RAW 264.7 macrophages in passage 9-12 were cultured in 12 well plates using DMEM (Invitrogen)
154 enriched with 10% fetal calf serum, 10µg/ml Gentamycin (Invitrogen) and 2mM L-Glutamine (Invitrogen).
155 Macrophages were stimulated, using either IFN γ (10ng/ml; PeproTech, Rocky Hill, NJ), IL-13 (10ng/ml;
156 PeproTech), PGE2 (5µM; PeproTech), or FDE at the concentrations of 1µg/ml, 50µg/ml and 100µg/ml for
157 48 hours.

158 After stimulation, cells were collected for the flow cytometry analysis. Cells were incubated with a 4
159 mg/ml Lidocaine, 10 mM EDTA and 10% fetal calf serum in phosphate-buffered saline solution (pH7.3) for
160 15 minutes. They were detached by vigorous pipetting, and transferred into 1,5 ml tubes. Cells were
161 washed 3 times with PFE before they were used for flow cytometry.

162 **Flow cytometric analysis of RAW cells**

163 Membrane staining was performed using APC/Cy7-conjugated anti-MHC class II antibody (Biolegend, San
164 Diego, USA) and AlexaFluor 647-conjugated anti-CD206 antibody (Biolegend) with a 30-minute incubation
165 in the dark, on ice. After washing for 3 times using cold PFE, cells were fixed and permeabilized for 30
166 minutes in cold Fix/Perm buffer (BD Biosciences) and then washed with cold permeabilization buffer (BD
167 Biosciences). After that, cells were incubated for 30 minutes in the dark with PE-conjugated anti-IL-10
168 antibody (eBioscience, Vienna, Austria) for intracellular staining. After the incubation, cells were washed
169 twice in permeabilization buffer and then were resuspended in PFE. An appropriate isotype control was
170 used for the IL-10 staining (rat IgG2b κ -PE, eBiosciences).

171 Analysis was performed using a BD FACSArray cytometer (BD Biosciences) and data was analyzed using
172 FlowJo software (Tree Star Inc., Ashland, USA). Mean fluorescence intensity (MFI) was used to evaluate
173 the expression of the different markers.

174 **Microscopy of RAW cells**

175 Cell cultures were photographed before each flow cytometric analysis, to observe changes in morphology
176 that could be related to changes in the milieu.

177 Pictures of the cells were made using an Olympus IX50 microscope (Olympus, Tokyo, Japan). Images were
178 manually focused around the middle point between the center of the well and the border of it. The

brightness, contrast and the color saturation settings were automatically calibrated by the capture program Cell^A (Olympus, Tokyo, Japan), which was also used to obtain the images.

Statistical methods

Nonparametric Kruskal-Wallis test was used to assess whether differences between groups existed, and when $p < 0.05$, Mann–Whitney *U*-test was performed to assess differences between subgroups. All analyses were performed using GraphPad Prism 5.0, and differences were considered statistically significant at two-sided *p*-values less than 0.05. $P < 0.10$ was considered a statistical trend.

Results

HDM and FDE induce differential types of activated CD11c⁺/CD11b⁺ alveolar macrophages in the lungs

To identify macrophage phenotypes, 6-color FACS analysis was performed. Macrophages were first identified based on autofluorescence and F4/80 expression. Total numbers of macrophages were higher in mice exposed to HDM as compared to PBS-exposed mice, and this number was even higher in FDE-exposed mice (figure 1A). Alveolar macrophage numbers, identified by high auto fluorescence and CD11c expression were also higher in HDM-exposed mice in comparison to PBS-exposed mice, and even further increased in FDE-exposed mice (figure 1B). Alveolar macrophages are known to lack expression of the adhesion molecule CD11b, except when they are activated or represent recruited macrophages (8). Activated alveolar macrophages, defined as CD11c⁺CD11b⁺ were previously shown to be increased after repetitive organic dust exposure (19). Within the CD11c⁺ positive population, we found that numbers of cells expressing CD11b were higher in HDM-exposed mice than in control mice and even higher in FDE-exposed mice.

To determine the type of tissue signals these activated alveolar macrophages have seen in each type of inflammation, we analyzed the expression of intracellular adhesion molecule 1 (ICAM-1), a marker of M1-dominant macrophages (8) and CD206 (mannose receptor), a marker of M2-dominant macrophages (26). HDM exposure induced higher expression of CD206 in CD11c⁺CD11b⁺ cells, while both HDM and FDE induced ICAM-1 expression in these cells, as compared to control mice. Of note, FDE exposure induced higher expression of ICAM-1 than HDM exposure but did not induce CD206 expression (figure 1D and E). These results demonstrate that although numbers of total macrophages, alveolar macrophages and activated alveolar macrophages are increased in different types of lung inflammation, the phenotype, and thus the function of the macrophages may be different depending on the type of inflammation.

Predominant macrophage phenotypes vary according to the type of inflammation

To further study the presence of the different macrophage phenotypes in lung tissue after HDM and FDE exposure, lung sections were stained for macrophages with a general macrophage marker (Mac3) in combination with more specific markers for macrophage subsets. M1-dominant macrophages were

identified as IRF-5⁺Mac3⁺ cells, M2-dominant macrophages were identified as YM1⁺Mac3⁺ cells and anti-inflammatory macrophages were characterized as IL-10⁺Mac3⁺ cells. Interestingly, although HDM-exposed mice had more M1-dominant macrophages in the lungs than PBS-exposed mice, FDE-exposed mice had the highest numbers of M1-dominant macrophages of all groups (figure 2A). In contrast, M2-dominant macrophage numbers were the highest in lungs of mice exposed to HDM, whereas FDE-exposed mice still had more M2-dominant macrophages than PBS control mice (figure 2B). Anti-inflammatory macrophage numbers were significantly lower in lungs from both HDM- and FDE-exposed mice, as compared to PBS-exposed mice (figure 2C).

Furthermore, cytokines were measured in lung homogenates and we found that levels of the M1-related cytokines TNF α and IL-1 β were higher in lung tissue of FDE-exposed mice than in PBS- and HDM-exposed mice (figure 3A and B). YM1 levels were increased in HDM-exposed mice as compared to PBS and FDE-exposed mice (figure 3C). IL-10 levels were increased in both HDM- and FDE-exposed mice as compared to control mice (figure 3D), suggesting that the main source of IL-10 in these mice lungs is not macrophages.

To confirm that the HDM and FDE models have induced Th2 and Th17 lung inflammation, Th2 cytokines and IL-17 were also measured in lung tissue. Levels of IL-4, IL-5 and IL-13 were significantly higher in HDM-exposed mice as compared to PBS- and FDE-exposed mice. IL-17A levels were the highest in FDE-exposed mice as compared to both HDM- and controls. HDM also had more IL-17A than PBS mice. Since it has been shown that FDE exposure can induce Th1 cytokines in mouse lungs (18), levels of IL-12p70 and IFN γ were also measured. Levels of IL-12p70 were higher in HDM-exposed mice as compared to PBS-exposed mice. With respect to IFN γ , similar levels were found in the different groups (table 1).

Macrophages polarize into M1-dominant, M2-dominant or anti-inflammatory subsets depending on the *in vitro* cytokine stimulation

To evaluate the polarization of macrophages *in vitro*, RAW cells were incubated for 48 hours with IFN γ , IL-13 and PGE2 to induce M1-dominant, M2-dominant and anti-inflammatory phenotypes respectively. Polarization of macrophages into the different phenotypes was assessed by flow cytometric analysis of MHC class II (M1-dominant), CD206 (M2-dominant) and IL-10 (anti-inflammatory). IFN γ stimulation

241 induced MHC class II expression (trend $p=0.0571$, figure 4A), while IL-13 and PGE2 stimulation induced
242 CD206 expression (figure 4B) and IL-10 expression (figure 4C).

243

244 **Farm dust induces M1-dominant, but not M2-dominant or anti-inflammatory macrophages *in vitro***

245 RAW cells were stimulated with FDE in concentrations of 1 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ to investigate
246 the direct effect of FDE exposure on macrophage polarization. FDE stimulation dose-dependently induced
247 MHC class II expression, whereas CD206 or IL-10 expressions were not induced (figure 5A, B and C).

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249

250

Discussion

This study demonstrates that in response to different types of exposures, different types of macrophages are present in the lungs, and that the predominant phenotype varies according to the type of inflammation. In our FDE model of nonallergic lung inflammation, the M1-dominant phenotype predominates, while in the HDM model of allergic inflammation M2-dominant macrophages are more prevalent. Furthermore, we showed that *in vitro* FDE exposure of RAW macrophages directly induced M1-dominant polarization, while it did not induce M2-dominant or anti-inflammatory macrophages. Two established models of lung inflammation were used to study the relative distribution of macrophage phenotypes: one model of HDM-induced allergic lung inflammation and one FDE model of nonallergic lung inflammation. Allergic inflammation relates typically to a Th2-type response, with high numbers of eosinophils in the lungs, high levels of serum IgE and Th2 cytokines. In contrast, FDE exposure was shown to induce chronic infiltration of Th1/Th17/Tc17 cells and lung neutrophilia in the mouse (18, 25). A common feature of both models is the presence of increased numbers of macrophages in the lungs (4, 22). Here we show that, comparing both models, the FDE model has larger numbers of total macrophages in the lungs, larger numbers of CD11c⁺ macrophages, and larger numbers of activated CD11c⁺/CD11b⁺ macrophages than is found in the HDM model. Of interest, when we focused on the phenotype of these cells, we found that macrophages differ in phenotype depending on the type of exposure and inflammation. Nonallergic inflammation was accompanied by higher numbers of macrophages with the M1-dominant phenotype, while the M2-dominant phenotype was relatively more present in allergic inflammation. The distinctive macrophage populations in our study were identified using specific markers for each subset. By means of flow cytometry, ICAM-1 expression was used to identify M1-dominant macrophages (8) and CD206 expression to identify the M2-dominant subset. Since the mannose receptor (CD206) is expressed by both M2-dominant and anti-inflammatory macrophages, an additional marker is required to identify both subsets. This was accomplished by immunohistochemistry, where double stainings were performed for Mac3 (general macrophage marker) in combination with IRF5, YM1 or IL-10, to identify M1-dominant, M2-dominant and anti-inflammatory macrophages respectively. IRF5 has been shown to be a

specific marker for M1-dominant macrophages *in vivo* and *in vitro* (9, 27), inducing transcription of genes encoding IL-12, IL-23 and suppressing the gene encoding IL-10 in diverse disease settings. YM1 is a unique marker for M2-dominant macrophages in the lung (4, 23). To identify anti-inflammatory macrophages, also known as regulatory macrophages, we chose to investigate the expression of the immunosuppressive cytokine IL-10, which is the most important characteristic of this type of macrophages (14). Considering that IL-10 can be produced by different cell types, the use of a double staining with Mac3 was pivotal to characterize anti-inflammatory macrophages.

We found increased numbers of IRF5-expressing macrophages after FDE exposure. Since the transcription factor IRF5 has been shown to directly activate transcription of IL-12 and IL-23 in macrophages and subsequently promote T cell differentiation of Th1 and Th17 lineages (9), it is tempting to speculate that M1-dominant macrophages are involved in the initiation of the adaptive nonallergic immune response, as observed in individuals occupationally exposed to farm environments and in our mouse model of FDE exposure. On the other hand, a critical role of macrophages in downregulation of inflammatory responses has been shown by Poole and coworkers, after prolonged exposure to FDE from a swine facility. The authors showed that depletion of macrophages, before and during FDE exposure enhanced lung neutrophil infiltration (19). Although the different macrophages were not phenotyped in that study, induction of M1-dominant cytokines TNF α and IL-6 by FDE was greatly diminished after macrophage depletion, suggesting presence of, in particular, M1-dominant macrophages in the FDE model.

In HDM-exposed mice, higher numbers of M2-dominant macrophages and higher levels of its chemokine YM1 were found as compared to control or FDE-exposed mice, which is in agreement with previous studies (4, 11, 12). YM1 is also known as eosinophil chemotactic factor (ECF-L) and we have previously shown that YM1 levels in BALF strongly correlated with eosinophils in BALF (4). With respect to anti-inflammatory macrophages, the highest numbers of these cells were found in control mice, reproducing our previous findings (4). Strikingly, IL-10 levels were increased in both HDM- and FDE-exposed mice as compared to control mice, suggesting that the main source of IL-10 in these mice lungs is not macrophages. An obvious other candidate for this would be regulatory T cells, which are known IL-10

producers (24). We have previously shown increased numbers of regulatory T cells in HDM-exposed mice (4). Other candidates for IL-10 production are B cells, which will be subject of future studies in our models. Our findings in the mouse were confirmed *in vitro*, as FDE exclusively induced M1-dominant polarization, and suggest that FDE exposure *in vivo* exerts a direct effect on macrophages, that may be independent of other cell types or mediators. This direct effect occurs probably through activation of pattern recognition receptors (PRRs) on macrophages by microbial components present in the FDE. LPS, a major component of the cell wall of gram-negative bacteria is recognized by the PRR Toll-like receptor 4 (TLR4) and it is known to be present in FDE, as well as muramic acid, a peptidoglycan (PGN) derived predominantly from gram-positive bacteria, which activates TLR2 and Nucleotide oligomerization domain 2 (NOD2)(20, 21). Moreover, it has been shown in monocyte-derived macrophages, that FDE exposure induces TNF α production (17), which can induce M1-dominant polarization. In addition to TLR activation-induced TNF transcription, some TLR ligands can activate Toll/IL-1 receptor domain-containing-adaptor protein inducing IFN β (TRIF)-dependent pathways, resulting in IFN β production (29). This endogenously produced IFN β can replace the IFN γ produced by NK cells and T cells and induce M1-dominant polarization (14). Conversely, M2-dominant macrophages are not directly polarized by HDM. Mouse alveolar macrophages recovered from BALF and stimulated *in vitro* with *Dermatophagoides farinae* showed increase in TNF α , IL-6 and nitric oxide production, all markers of M1-dominant polarization (2). This shows that M2-dominant polarization is dependent of IL-4 and/or IL-13 produced by other cell types. Considering the diversity of macrophage functions, M1-dominant macrophages would be needed directly after FDE exposure due to their enhanced phagocytosis and antigen presentation capabilities to accomplish microbial clearance, while M2-dominant macrophages are instructed at a later stage by the damaged tissue for repair purposes.

The role of M2-dominant macrophages in allergic asthma is still an ongoing debate, but it is thought that these cells could contribute to disease through secretion of extracellular matrix components and attraction of eosinophils (6). Correlations between asthma severity and numbers of M2-dominant macrophages or their products in lungs and serum of asthmatic patients (3, 12) and in mouse models (4) indicate that these cells may actively contribute to disease. On the other hand, it has been shown in mice

with abrogated IL-4 receptor α signaling on macrophages, that the M2-dominant phenotype is not necessary for allergic lung inflammation and may be a consequence of an elevated Th2 response (16). The finding that anti-inflammatory macrophages were found in lower numbers in HDM- and FDE-exposed mice as compared to control mice indicates that this phenotype is prevalent in homeostasis and suggests that during inflammation polarization into other macrophage subsets occurs. One could speculate that once microbial clearance is accomplished, a shift towards anti-inflammatory phenotype takes place to achieve resolution of inflammation.

In conclusion, in both nonallergic and allergic models of lung inflammation high numbers of CD11c⁺/CD11b⁺ macrophages are induced in the lungs. The polarization status of these macrophages, however, is different depending on the environment, which determines the different types of inflammation in each model. In the nonallergic phenotype, the M1-dominant subset predominates, which may contribute via IRF5 to Th1-Th17 responses; while in allergic conditions, the M2-dominant phenotype is more prevalent. This information may be important in the identification of new targets for therapeutic interventions in which skewing of macrophage polarization could be used as treatment for lung inflammation.

347 Acknowledgements

348 The authors thank Annejet Spierenburg for the collection of farm dust samples, Jack Spithoven for the
349 preparation of the farm dust extract, Pieter Klok for his assistance with animal experiments and Catharina
350 Reker-Smith for her help with the RAW cell experiments.

351

352 Disclosures

353 The authors have no conflict of interest, financial or otherwise to disclose.

354

355 Funding

356 This work was supported by Grant nr 3.2.09.36 from the Netherlands Lung Foundation

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Legends

Figure 1: HDM and FDE induce different phenotypes of activated CD11c⁺/CD11b⁺ macrophages in the lungs. Mice were exposed to PBS, HDM or FDE, four times/week for 6 consecutive weeks and lung cells were isolated, stained for FACS analysis. Scatter dot plots showing median and distribution of (A) numbers of F4-80⁺/autoflu⁺ macrophages, (B) numbers of CD11c⁺ cells within macrophage population, (C) numbers of CD11b⁺ cells within the CD11c⁺ population. Mean fluorescence intensity (MFI) of surface markers (D) ICAM-1 and (E) CD206 on gated CD11c⁺CD11b⁺ cells are shown. Representative dot plots of lung cells of PBS-, HDM- and FDE-exposed mice are shown.

Figure 2: Predominant macrophage phenotypes vary with the type of exposure. Scatter dot plots showing median and distribution of (A) numbers of IRF5⁺Mac3⁺ cells (B) YM1⁺Mac3⁺ cells and (C) IL-10⁺Mac3⁺ cells in lung tissue of mice exposed to PBS, HDM or FDE four times/week for 6 consecutive weeks. Representative pictures of immunohistochemical stainings are shown for all three double stainings of PBS, HDM and FDE groups.

Figure 3: Scatter dot plots showing median and distribution of levels of (A) TNF α , (B) IL-1 β , (C) YM1 and (D) IL-10 in lung tissue of mice exposed to PBS, HDM or FDE four times/week for 6 consecutive weeks.

Figure 4: Macrophages polarize into M1-dominant, M2-dominant and anti-inflammatory macrophages in response to IFN γ , IL-13 and PGE2 stimulations, respectively. Bar graphs of mean fluorescence intensity (MFI) of (A) MHC II (M1-dominant), (B) CD206 (M2-dominant) and (C) IL-10 (anti-inflammatory) of RAW cells stimulated with IFN γ , IL-13 or PGE2 for 48 hours. Each bar displays the mean and SD of 4 independent experiments. Representative pictures of RAW cells are shown from control group, and IFN γ , IL-13 or PGE2 stimulated groups. *p<0.05 compared to control.

Figure 5: FDE induces M1-dominant, but not M2-dominant or anti-inflammatory macrophages. Bar graphs of expression of A) MHC II, B) CD206 and C) IL-10 of RAW cells stimulated with IFN γ , IL-13, PGE2 or three different concentrations of FDE for 48 hours. Each bar displays the mean and SD of 4 independent experiments. Representative pictures of RAW cells are shown from FDE and IFN γ stimulations showing that FDE stimulation induces morphologic changes similar to IFN γ . *p<0.05 compared to control.

Table 1: Th1, Th2 and Th17 cytokines in lung tissue

	PBS	HDM	FDE
Th1 cytokines			
IL-12p70	0.44 (0.13-0.67)	0.92 (0.40-1.84)*	0.46 (0.33-0.98)
IFN γ	1.75 (0.69-4.37)	2.52 (0.77-11.30)	1.55 (0.93-4.92)
Th2 cytokines			
IL-4	0.15 (0.12-0.28)	22.34 (7.21-51.07)***	2.56 (1.58-5.44)**###
IL-5	0.48 (0.28-1.39)	32.30 (7.17-122.20)***	1.47 (0.84-3.47)*###
IL-13	0.24 (0.11-5.07)	18.85 (8.66-82.69)***	2.23 (0.71-3.37)*###
Th17 cytokine			
IL-17	0.81 (0.35-3.45)	8.72 (1.67-23.49)***	47.14 (12.60-119.90)***###

Values are medians, pg/g tissue (range)

Subgroup analysis: HDM vs PBS or FDE vs PBS groups= *p<0.05; **p<0.01; ***p<0.001

HDM vs FDE groups= #p<0.05; ##p<0.01; ###p<0.001

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