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Altered innate immune response in farmers and smokers

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Pig farmers and cigarette smokers are continuously exposed to pathogen-associated molecular patterns (PAMPs) have an increased prevalence of respiratory disorders, such as chronic bronchitis and chronic obstructive pulmonary decease (COPD). We hypothesized that markers of innate immunity, T-helper (Th) cell cytokine profile and acute responses to pro-inflammatory stimuli differ between smokers and farmers, who are exposed to organic material on a daily basis and healthy non-exposed subjects. Eleven non-smoking pig farmers, 12 non-farming smokers and 12 controls underwent bronchial lipopolysaccharide (LPS) challenge and exposure in a pig barn during 3 h on separate days. Toll-like receptor 2 (TLR2), TLR4 and CD14 on blood monocytes and neutrophils and intracellular cytokine profile of Th cells were assessed before and 7 h after exposures. The same outcomes were analysed on peripheral blood and purified neutrophils from farmers and controls after stimulation *ex vivo* with dust from a pig barn and LPS. Circulating neutrophils and IL-13 and IL-4 producing Th cells were increased in smokers and farmers and TLR2 expression on blood monocytes was decreased in farmers compared with controls and smokers. After *in vivo* exposure, altered TLR expression was only observed in controls and the *ex vivo* stimulations showed an attenuated response in farmers compared to the control group. The inflammatory systemic response to pro-inflammatory stimuli is altered in farmers and smokers probably because of adaptive mechanisms arising from chronic exposure to organic material. This increased proportion of Th2 cells and reduced TLR2 expression may have health-related implications and may be related to the increased prevalence of respiratory disorders observed in these groups.

Keywords: Toll-like receptors, cytokines, helper T-cells, inflammation, organic dust, lipopolysaccharide, peptidoglycan, farmer, cigarette smoker

Abbreviations: TLRs, Toll-like receptors; PRRs, pattern-recognition receptors; PAMPs, pathogen-associated molecular patterns; Th, T-helper; IFNs, interferons; IRFs, interferon regulatory factors, LBP, lipopolysaccharide binding protein; PGN, peptidoglycans; COPD, chronic obstructive pulmonary disease

Introduction

Recognition of micro-organisms is an important function of the innate and adaptive immunity and pattern-recognition receptors (PRRs) recognize a variety of pathogen-associated molecular patterns (PAMPs) from viruses, bacteria, fungi and parasites.[1] Toll-like receptors (TLRs) comprise a group of PRRs which are expressed by most immune cells. When binding to PAMPs, immune responses including expression of pro-inflammatory cytokines and type I interferons (IFNs) (IFN-α and IFN-β) are mediated through activation of the transcription factor NF-κB and interferon regulatory factors (IRFs). Currently, eleven TLRs have been

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identified in human of which TLR2 and TLR4 are the most studied. Toll-like receptor 4 acts in a receptor complex together with CD14, the adapter molecule MD-2 and lipopolysaccharide binding protein (LBP), to bind lipopolysaccharide (LPS) from Gram-negative bacteria[1,2] and TLR2 recognizes PAMPs from Gram-positive bacteria like peptidoglycans (PGN) and lipoproteins. In addition, TLR2 recognizes and binds LPS from special bacteria strains and PAMPs from fungi.[1,2]

Pig confinement buildings contain high amounts of airborne inhalable organic dust up to a concentration of 25 mg/m^3.[3] Apart from particles from hay, grasses, pollen, epithelial cells (from pigs) and feedstuff, the dust contains high amounts of Gram-positive and Gram-negative bacteria and fungi,[4] as well as high levels of airborne LPS and PGN.[3] In previously unexposed healthy subjects, 3-h exposure in a pig confinement building leads to influenza-like symptoms such as fever, cough and malaise and a more than 3-fold increase in bronchial responsiveness to metacholine. In addition, exposure induces an intensive upper and lower airway inflammation with release of pro-inflammatory cytokines into the airways and influx of inflammatory cells, in particular neutrophilic granulocytes.[5–8] However, there are findings indicating that pig farmers, who are continuously exposed to organic dust, have an altered inflammatory response to acute exposure in a pig barn compared to healthy, previously unexposed subjects. Thus, adaptive mechanisms and development of tolerance may occur following repeated exposure to organic dust in a pig barn, a phenomenon that has also been described following repeated exposure to LPS.[9,10] Thus, in a small study by Palmberg *et al.*,[11] an increase of neutrophilic granulocytes in the upper airways and increased serum levels of interleukin (IL)-6 following exposure in a pig house was shown in healthy previously unexposed subjects but not in pig farmers. Although daily exposure in the farming environment attenuates the acute inflammatory response to pig barn dust, epidemiological studies have shown that pig farmers, who are regularly exposed in pig barns, have a higher prevalence of respiratory disorders such as chronic bronchitis and chronic obstructive pulmonary disease (COPD) than the population in general.[12–14] It is well known that these conditions are also frequently occurring among smokers.

Smokers are, similar to farmers, continuously exposed to PAMPs. Smoke from cigarettes contains high levels of bioactive LPS.[15,16] Droemann *et al.*[17] recently showed that TLR2 expression on alveolar macrophages is down-regulated in smokers and in patients with COPD, which may contribute to the impaired defence against respiratory infections observed in COPD. It has also been shown that smoking alters the profile of T-helper (Th) cells, favouring a Th2 cells,[18–20] which also has been observed after inhalation of LPS.[21]

The aim of the study was to compare the expression of TLR2, TLR4 and CD14 on peripheral blood monocytes and neutrophils and the cytokine profile of peripheral blood Th lymphocytes in two groups with repeated exposure to organic material (pig farmers and smokers) and healthy, non-smoking non-farmers before and after acute exposure in a pig house and a LPS challenge. The aim was also to investigate the expression of TLR2, TLR4 and CD14 and release of pro-inflammatory cytokines following *ex vivo* stimulation of peripheral blood and purified neutrophils from farmers and smokers with LPS and dust collected from a pig barn. Our hypothesis was that exposure to pathogen-associated molecular patterns (PAMPs) on a daily basis (farmers and smokers) alters the innate immune response.

Materials and Methods

Subjects and study design

Thirty six subjects were included in the study; 12 non-smoking pig farmers (2 females), mean age 41 y (range, 22–61 y), 12 smokers (2 females) mean age 41 y (range, 25–58 y) and 12 non-smoking, non-farming controls (2 females), mean age 33 y (range, 25–54 y). All subjects were non-atopic (verified by a negative skin prick test to 15 common allergens), had no history of asthma or COPD and had normal lung function assessed by spirometry performed according to the ATS recommendations.[22] The pig farmers had worked for 13 y (range, 0.5–36 y), 3.5 h/d (range, 0.5–8 h/d) in pig barns containing 1000 pigs (range, 65–3200 pigs). The smokers had a cumulative exposure of 21 pack-years (range, 1.5–48 pack-years) and they had smoked at least 10 cigarettes per day during recent years.

All subjects underwent a 3-h exposure in a barn while helping a farmer weighing pigs. Participants from all three groups were exposed at each occasion. On a separate day, LPS challenge was performed by inhaling a LPS solution with an inhalation dosimeter (SPIRA® Elektro 2, Hameenlina, Finland). Pig barn exposure and LPS challenge were performed in random order with a minimum of 3 weeks in between. The LPS solution was prepared from *Escherichia coli* serotype O111 : B4 (Sigma) dissolved in sterile saline (9 g/l) at a concentration of 1.25 mg/ml. The subjects inhaled 6 breaths from the dosimeter, corresponding to a LPS dose of 53.4 μg. Peripheral blood was drawn approximately 2 weeks before the first exposure and 7 h after the start of pig house and LPS exposures.

On the day prior to the first exposure, whole blood and purified neutrophils from farmers and non-smoking controls were stimulated *ex vivo* with *E. coli* LPS or

dust collected in a pig barn. The study was approved by the Karolinska Institute Ethics Committee (Stockholm, Sweden), Protocol No. 03-739, and all individuals included in the study gave their informed consent to participate.

Respiratory and inhalable dust measurements

Portable pumps with IOM filter cassettes (25 mm; SKC Ltd, Dorset, UK) and plastic cyclones (25 mm; Casella Ltd, London, UK) were used to monitor inhalable and respiratory dust levels, respectively. The samplers were placed in the breathing zone on 1 or 2 subjects at each exposure occasion and sampling was performed at an airflow of 1.9–2.0 l/min. The airflow was measured before and after the sampling was completed and an average value of the airflow was used for calculation of sampled air volume. The cassettes were equipped with Teflon filters (1.0 μm; Millipore, Sundbyberg, Sweden). After weighing, the filters were extracted and the endotoxin concentration was analysed by the use of a kinetic technique version of *Limulus amebocyte* lysate assay (Endosafe® Endochrome-K™ US Licence No. 1197; Coatech AB, Kungsbacka, Sweden), with *E. coli* O111 : B4 as standard.

Peripheral blood sampling

Whole peripheral blood was collected in ethylene diamine-tetra-acetic acid (EDTA) Vacutainer tubes (BD Bioscience, San Jose, CA, USA) for assessing cell surface markers and in heparinized tubes (BD Bioscience) for *ex vivo* stimulation and intracellular cytokine staining.

Purification of neutrophils

A Vacutainer tube with 10-ml heparinized peripheral blood was centrifuged to discard the plasma. Blood cells were mixed with PBS containing 2% dextran (1 : 1) and left standing 30 min for sedimentation of red blood cells. Upper phase with leukocytes was divided into two new tubes and washed with phosphate buffer solution (PBS). The cell pellets were mixed with 4.5 ml of lysing buffer and incubated for 15 min. After the incubation 4.5 ml of Lymphoprep™ (Axis-Shield, Norway) was gently added to the bottom of the tubes (under the cell suspension) and centrifuged for 40 min without braking. The pellets were washed with MACS buffer (PBS with 2% bovine serum albumin [BSA] and 2 mM EDTA, pH 7.2). To isolate the neutrophils, the cells were incubated with magnetically labelled MACS® anti-CD16 (Milteniy Biotech, Auburn, CA, USA) for 30 min at 4°C. The magnetically labelled $CD16^+$ cells were isolated following the manufacturer's instructions using a magnet and LS columns (MiniMACS, Milteniy Biotech). The $CD16^+$ cells were washed and suspended in RPMI 1640 culture media (Sigma–Aldrich).

Stimulation of whole blood and purified neutrophils

Heparinized peripheral blood or purified neutrophils were incubated together with RPMI 1640 containing 2 mmol/l glutamine (Sigma–Aldrich) for 2 h in 37°C. During the 2-h incubation, blood was stimulated with *E. coli* LPS or organic dust from one pig barn (collected in the pig barn on shelves and window ledges about 1.2 m above the floor, where the dust are settled in high amounts).

Cell distribution

To determine the absolute cell number in peripheral blood before and after exposure, TruCOUNT™ tubes containing a specified number of beads were used. Whole blood and a four–color antibody mixture (CD3 FITC/CD8PE/CD45PerCp/CD4APC from BD Bioscience) were added to a TruCOUNT tube (BD Bioscience) and incubated in the dark at 20–22°C for 15 min. To lyse red blood cells, 450 μl BD PharM Lyse™ (BD Bioscience) was added and an additional 10 min incubation, in the dark at 20–22°C, was performed. All samples were then analysed on a FACSCalibur™ (BD Bioscience) using a set made in Attractor™ (BD Bioscience) to perform a five-part white blood cell differential. The results are presented as number of cells per volume blood.

Toll-like receptor and CD14 staining

To measure total amount of TLR2, TLR4 and CD14 expressed on monocytes and neutrophils, surface staining and flow cytometry analyses were performed. Whole blood, stimulated blood cells or purified neutrophils were stained with titrated amounts of either anti-TLR2 PE (TLR2.1; eBioscience, San Diego, CA, USA) or anti-TLR4 PE (HTA125, eBioscience) together with anti-CD14 FITC (61 D3, eBioscience) and CD45 PerCp (BD Bioscience Pharmingen). As negative controls, isotype-matched antibodies were used. Red blood cells were lysed with BD PharM Lyse™ (BD Bioscience Pharmingen). Cells were then washed with PBS containing 1% fetal calf serum (FCS) (Sigma Aldrich) and resuspended in CellFIX™ (BD Bioscience). Samples were analysed using FACSCalibur™ (BD Bioscience) flow cytometer and CELLQuest™ (BD Bioscience).

The samples were run on separate days and the flow cytometer was calibrated every day before use. To gate on monocytes and neutrophils, forward scatter and side scatter were used (Fig. 1A). The results are presented as relative median fluorescence intensity (rMFI = monoclonal antibody/matched isotype control).

Whole blood stimulation and intracellular cytokine staining

Heparinized peripheral blood was incubated in RPMI 1640 containing 2 mmol/l glutamine (1 : 1 dilution) and GolgiPlug (Brefeldin A; BD Bioscience) for 4 h at 37°C. During the 4-h incubation, blood was stimulated with a combination of 25 ng/ml of phorbol 12-myristate 13-acetate (PMA) and 1 μmol/l of ionomycin (Sigma–Aldrich). After stimulation, the blood cells were washed and resuspended in staining buffer (PBS with 1% FCS). Cells were directly stained with anti-CD4 APC and anti-CD3 PerCp monoclonal antibodies (BD Bioscience) for 20 min in the dark and at 20–22°C. To lyse red blood cells, BD PharM Lyse™ (BD Bioscience Pharmingen) was added and 10 min incubation in the dark and at 20–22°C was performed. After washing with staining buffer, cells were incubated with Cytofix/Cytoperm™ (BD Bioscience Pharmingen) for 20 min at 20–22°C. After centrifugation, the cells were resuspended and incubated for 10 min in the dark, at 20–22°C with Perm/Wash™ (BD Bioscience Pharmingen). Intracellular cytokines were then stained for 30 min in the dark, at 20–22°C with anti-IL-4 PE, anti-IFN-γ FITC, anti-IL-2 FITC

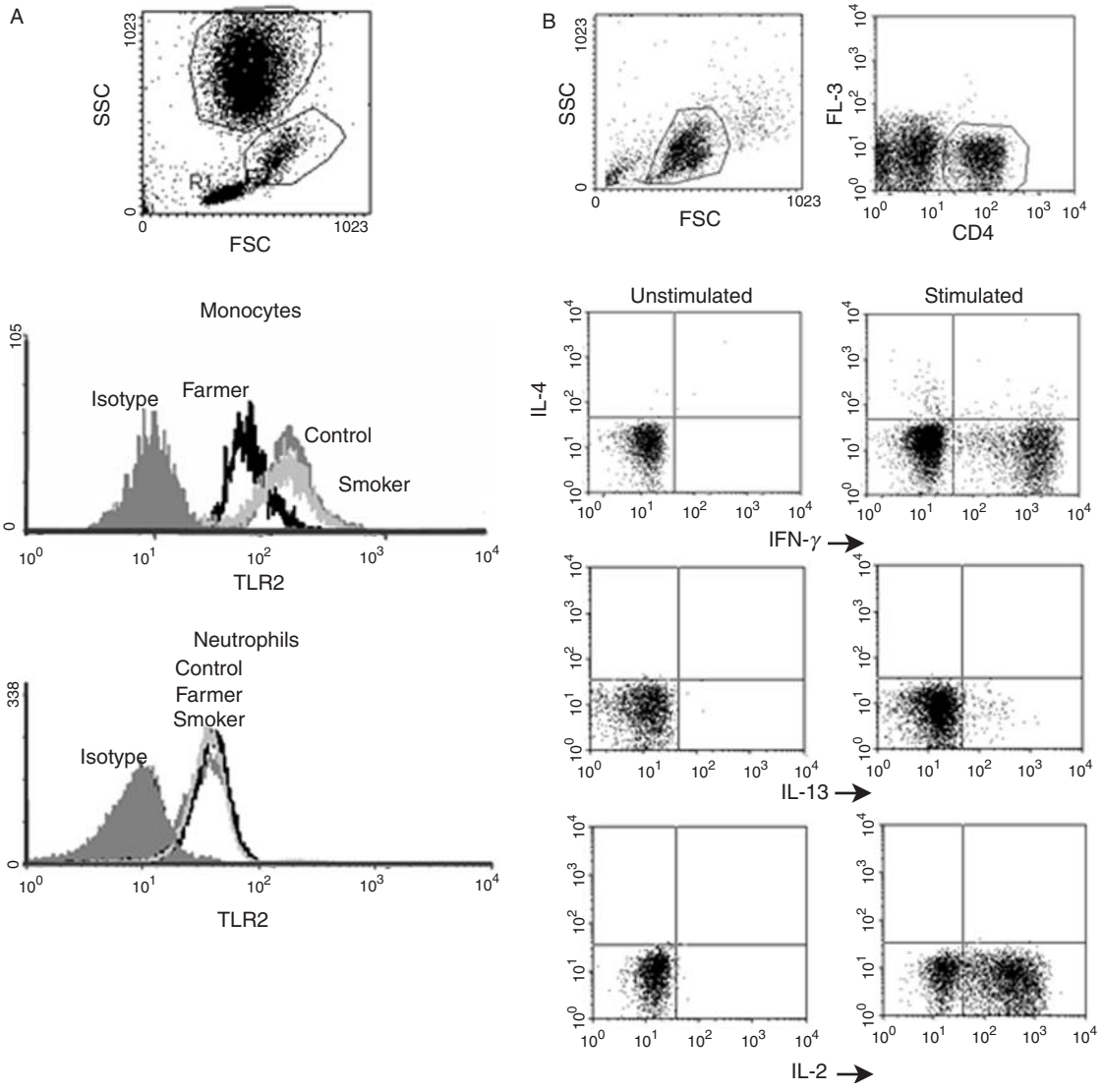

Fig. 1. Pattern-recognition receptor surface expression and intracellular cytokines was determined by flow cytometry. Dot plot showing gates on monocytes and neutrophils in peripheral blood and representative histogram of TLR2 expression on peripheral blood monocytes and neutrophils (A). Representative dot plots showing IFN-γ, IL-4, IL-2 and IL-13 production by unstimulated and stimulated peripheral blood T-helper cells (B).

(BD Bioscience) and anti-IL-13 PE (BD Bioscience Pharmingen). Cells were washed with Perm/Wash and resuspended in CellFIX™ (BD Bioscience). Unstimulated blood was also incubated and stained with the same procedure as negative control. Flow cytometric acquisition and analysis was performed using FACSCalibur™ (BD Bioscience) flow cytometry and CELLQuest™ software (BD Bioscience). To gate on Th cells, forward scatter, side scatter, and CD4 where used, and IL-2, IL-4, IL-13 and IFN-γ positive cells were gated by the use of unstimulated stained blood (Fig. 1B). The results were expressed as percentage of IL-2, IL-4, IL-13 and IFN-γ cytokine-producing Th cells in a total population of Th cells.

Interleukin-6 in serum

Interleukin-6 was measured in serum using high-sensitivity ELISA kit (R&D systems, Europe, Abingdon, UK) as suggest by the manufacturer. The detection range for IL-6 was 0.156–10 pg/ml.

Interleukin-6 and IL-8 release after ex vivo *stimulations*

Interleukin-6 and IL-8 were measured in the supernatants with an in-house ELISA method.[23] Commercially available antibody pairs (R&D systems, Europe) were used. The detection ranges for IL-6 and IL-8 were 2.8–375 pg/ml and 40–3200 pg/ml, respectively. For duplicate samples, an intra-assay coefficient (CV) was set to be <10%.

Statistical analysis

Data are given as mean (±SEM) unless otherwise stated. Statistical analyses were performed by using simple regression and ANOVA with Fisher's PLSD (between group comparisons) and paired *t*-tests (within group comparisons) as *post hoc* tests when appropriate. As IL-6 data in serum had a very skewed distribution, data were analysed by using Friedman and Wilcoxon Signed Rank (within group comparison) and Kruskal–Wallis and Mann–Whitney (between group comparisons). A *P*-value of <0.05 was considered significant.

RESULTS

One of the pig farmers was pregnant at the time of the first examination and was, therefore, excluded from all *in vivo* exposures. In addition, *in vivo* PRR-results are missing from one farmer due to technical problems.

Exposure measurements

The airborne levels of inhalable and respirable dust in the pig barns were 8 mg/m^3 (6–10 mg/m^3; median 25 th–75 th percentiles) and 0.32 mg/m^3 (0.30–0.33 mg/m^3), respectively. The corresponding endotoxin concentrations were 63 ng/m^3 (48–85 ng/m^3) and 13 ng/m^3 (3–27 ng/m^3). Exposure data have also been presented elsewhere.[24]

Pre-exposure analyses

The number of circulating neutrophils was higher in farmers ($P = 0.019$) and smokers ($P = 0.002$) than in controls (Fig. 2A) and circulating monocytes was higher in smokers than in controls ($P = 0.004$) and farmers ($P < 0.001$; Fig. 3A). The TLR2 expression on blood monocytes was lower in farmers than in controls ($P = 0.003$) and smokers ($P = 0.038$; Fig. 3B).

Compared with controls, farmers and smokers had higher percentage of Th cells expressing IL-13 ($P = 0.04$; Fig. 4B) and IL-4 ($P = 0.002$; Fig. 4C). There were no significant differences between the groups regarding the Th cell expression of IL-2 and IFN-γ (Fig. 4D,E). The proportion of IL-13 producing Th cells correlated with the cumulative exposure in pig farmers ($R^2 = 0.533$; $P = 0.011$; Fig. 5). No relation between IL-13 producing cells and cumulative exposure to tobacco (pack-years) was observed among smokers. Smokers had increased level of IL-6 in serum compared with controls ($P < 0.001$; Fig. 6).

Post-exposure analyses

Leukocytes

The peripheral blood concentration of neutrophils increased significantly after exposure in the pig barn and after LPS challenge in all three groups (Fig. 2A). The LPS-induced blood neutrophilia in smokers exceeded that of farmers and controls ($F = 7.652$; $P = 0.002$; Fig. 2A). Monocyte concentration in blood increased in all groups after exposure in the pig barn and after LPS challenge, with no significant differences between the groups (Fig. 3A). There were no significant changes regarding concentration of blood lymphocytes (Fig. 4A), basophils and eosinophils (data not shown) after exposure in the pig barn and after LPS challenge.

Toll-like receptor 2, TLR4 and CD14

Toll-like receptor 2 expression on blood monocytes was not altered by exposures and TLR2 expression on neutrophils did not change after pig house exposure in any group. However, the TLR2 expression on neutrophils increased significantly after LPS challenge in controls ($P = 0.041$); the increase being significantly greater than in smokers and farmers ($F = 7.132$;

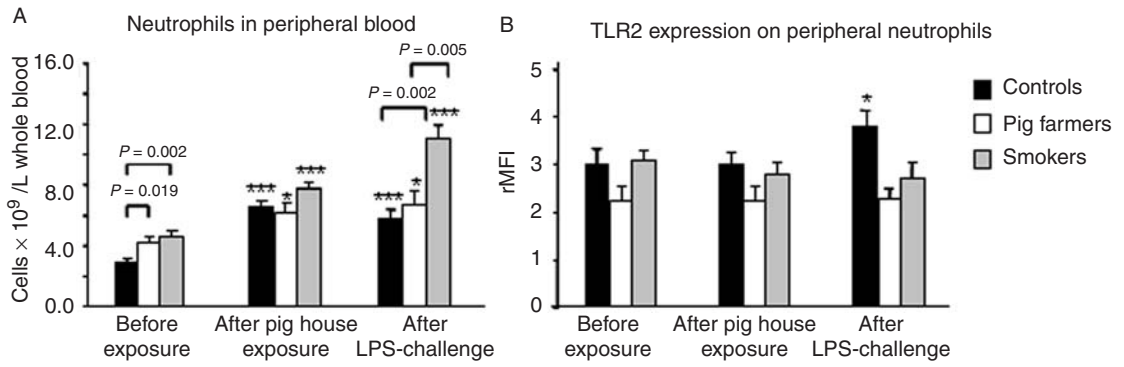

Fig. 2. Concentration of neutrophils in peripheral blood (A) and surface expression of TLR2 on peripheral blood neutrophils (B), before and after exposure in a pig barn and a LPS challenge ($n = 10$–12). $^{*}P < 0.05$, $^{***}P < 0.001$, compared with pre-exposure values. Brackets indicate pre-exposure differences between the groups or differences in exposure induced changes between the groups. rMFI = relative mean fluorescence intensity.

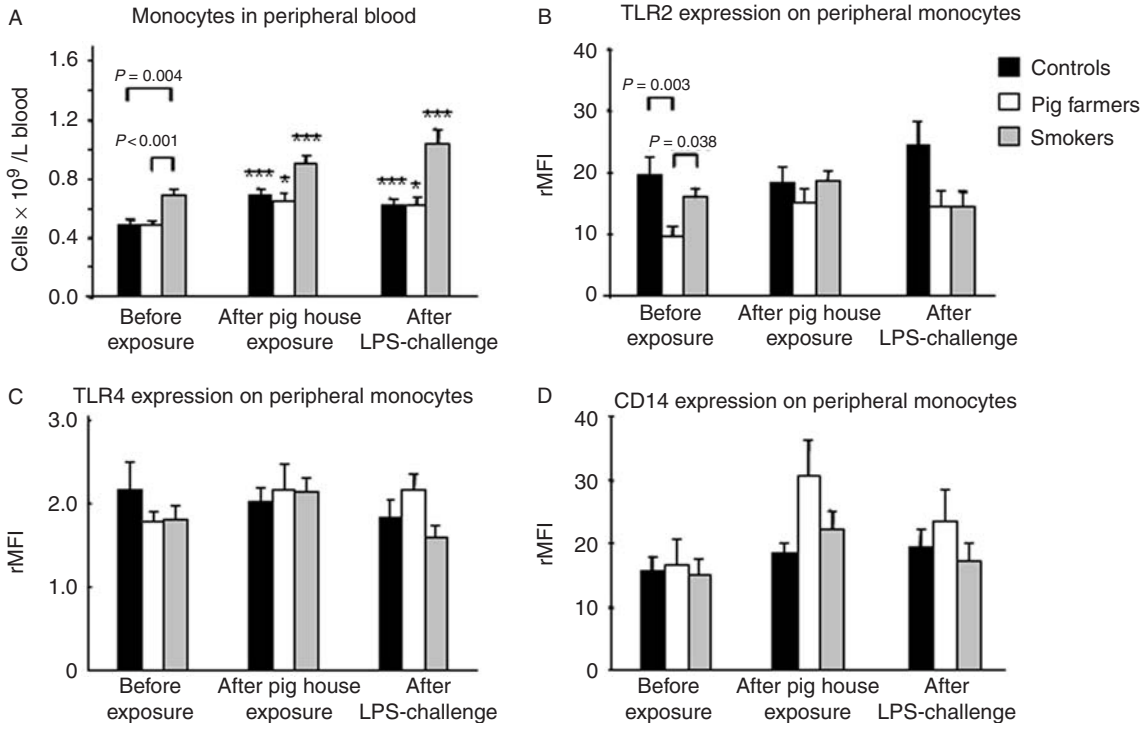

Fig. 3. Concentration of monocytes in peripheral blood (A) and surface expression of TLR2 (B), TLR4 (C) and CD14 (D) on peripheral blood monocytes, before, after exposure in a pig barn and after LPS challenge ($n = 10$–12). $^{*}P < 0.05$, $^{***}P < 0.001$, compared with pre-exposure values. Brackets indicate pre-exposure differences between the groups or differences in exposure induced changes between the groups. rMFI = relative mean fluorescence intensity.

$P = 0.003$; Fig. 2B). There were no significant changes in TLR4 and CD14 expression after pig house exposure and LPS-challenge, either on monocytes (Fig. 3B–D) or on neutrophils. However, the expression of TLR4 and CD14 on neutrophils was very weak (very close to background staining) and, therefore, not valid to draw firm conclusions.

Intracellular IL-2, IL-4, IL-13 and IFN-γ

The proportion of IL-4 and IL-13 producing Th cells (of total Th cell population) increased in controls after pig house exposure and after LPS challenge with no differences between the groups (Fig. 4B,C). The percentage of Th cells producing IFN-γ also increased in controls after LPS challenge with no difference between the groups (Fig. 4E). There was no significant change of intracellular IL-2 expression after exposure in either group (Fig. 4D).

Interleukin-6 in serum

After exposure in the pig house there was a significant increase of serum IL-6 in smokers and controls; the

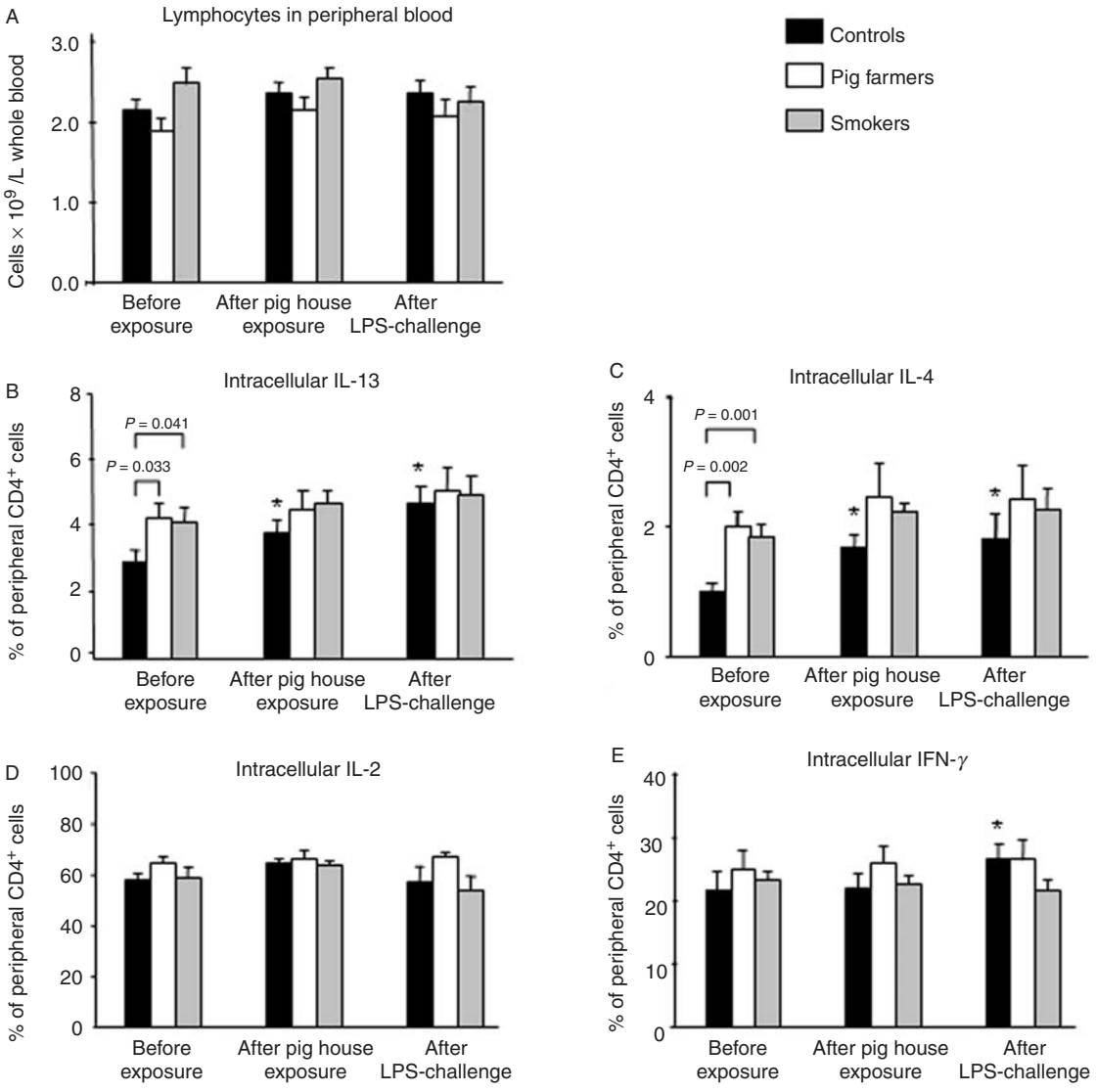

Fig. 4. Concentration of lymphocytes in peripheral blood (A) and percentage of peripheral T-helper cells producing intracellular IL-13 (B), IL-4 (C), IL-2 (D) and IFN-γ (E), before, after exposure in pig barn and after LPS challenge ($n = 10$–12). *$P < 0.05$, compared with pre-exposure values. Brackets indicate pre-exposure differences between the groups.

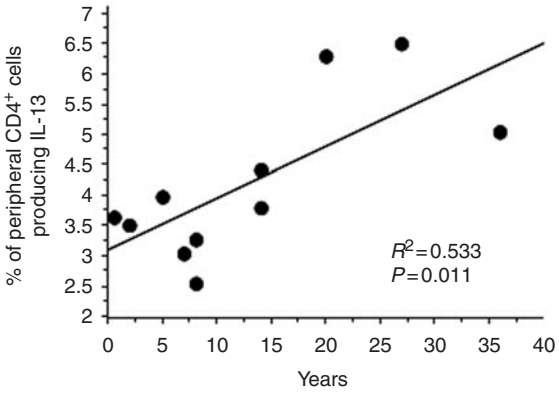

Fig. 5. Correlation between percentage of T-helper cells producing IL-13 and cumulative exposure (number of years the farmers had been working in a pig barn; $n = 11$).

increase in controls being higher than in farmers ($P = 0.010$; Fig. 6). After LPS challenge, there was an increase of IL-6 in all three groups, and the increase in smokers was higher than in famers ($P = 0.007$) and controls ($P = 0.023$; Fig. 6).

Ex vivo *stimulations (farmers and controls)*

The expression of PRRs on neutrophils is low and neutrophils stimulated with pig barn dust exhibited intense autofluorescence (probably due to high content of glucans in the dust) which made it difficult to assess accurately PRR expression after dust stimulation. Dust-induced effects on neutrophils were, therefore, not included in the *ex vivo* study. Monocytes also exhibit

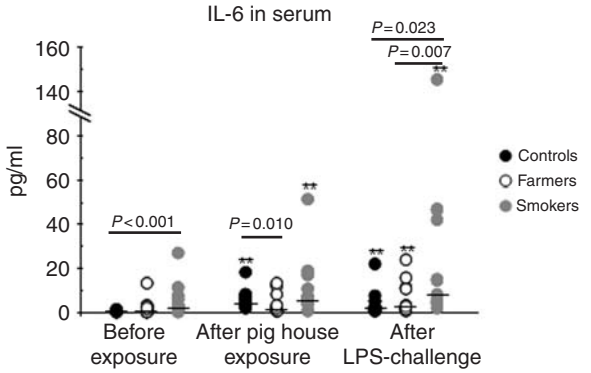

Fig. 6. Concentration of IL-6 in serum before and after exposure in a pig barn and a LPS challenge ($n = 11$–12). $^{**}P < 0.01$, compared with pre-exposure values. Brackets indicate pre-exposure differences between the groups or differences in exposure induced changes between the groups.

autofluorescence after pig barn dust stimulation but, by using isotype control together with unstained cells and due to the higher PRR expression on monocytes, the background was exceeded.

Toll-like receptor 2, TLR4 and CD14

Neither LPS nor dust stimulation influenced TLR2 expression on monocytes in farmers. In the non-farming control group the lowest dose of LPS ($P = 0.026$) and all three doses of dust increased TLR2 expression on monocytes ($P = 0.007$); the increase observed after dust stimulation being significantly higher in non-farmers than in farmers ($P = 0.030$; Fig. 7A,B). Lipopolysaccharide and dust did not influence the TLR4 expression on monocytes (Fig. 7C,D) but both stimuli increased the CD14 expression on peripheral blood monocytes dose dependently in both groups ($P < 0.001$) with no difference between the groups. However, farmers showed enhanced CD14 expression on monocytes with increased CD14 expression in media alone and in the lowest and highest dose of dust compared to controls ($P = 0.049$; Fig. 7 E,F). LPS did not influence TLR2 expression either on neutrophils from peripheral blood or on purified neutrophils (Fig. 8A,B). After *ex vivo* stimulations, the expression of TLR4 and CD14 on neutrophils was, as observed *in vivo*, very weak. No alteration in TLR4 and CD14 expression on neutrophils was observed, except in the highest doses of LPS-stimulated peripheral blood (no differences between the groups). However, as the expression was very close to background staining, the results are almost certainly too unstable to draw firm conclusions.

Interleukin-6 and IL-8 after ex vivo stimulation (farmers and controls)

After whole blood stimulation *ex vivo*, there was an increased release of IL-6 at the lowest dust concentration in controls ($P < 0.001$) and the increase was higher than in farmers ($P = 0.035$). Lipopolysaccharide stimulation did not influence the IL-6 release in either group (Fig. 9A,B). All concentrations of LPS and dust increased the release of IL-8 in controls and farmers with no difference between the groups (Fig. 9C,D).

Discussion

Pig farmers and smokers are continuously exposed to PAMPs known to activate receptors of the innate and adaptive immunity. In the present study, it was demonstrated that the expression of TLR2 on blood monocytes is down-regulated in farmers compared with smokers and non-smoking, non-farming healthy controls. It was also shown that TLR2 expression on blood cells following exposures *in vivo* and stimulation *ex vivo* is attenuated and that systemic IL-6 response in farmers was diminished compared to non-farming controls whereas *ex vivo* stimulation increased CD14 expression dose-dependently in both farmers and controls. Further, it was demonstrated that farmers and smokers have an increased proportion of IL-13 and IL-4 producing peripheral blood Th cells compared to the control group. Following exposure in the pig barn and to a bronchial LPS challenge, IL-13 and IL-4 expression of circulating T-lymphocytes was clearly enhanced in the controls whereas the expression was high already prior to exposure and not further increased in farmers and smokers.

A possible explanation for the attenuated TLR2 expression on circulating monocytes in pig farmers is the high daily exposure to TLR2 agonists such as peptidoglycan in pig barns. Pig farmers are frequently colonized with bacteria (often resistant to antibiotics), which might further influence expression of PRRs.[25] After *in vivo* exposure, there was an increased surface TLR2 expression on neutrophils in the controls after LPS challenge. We cannot exclude the possibility that changes may have occurred at other time points as we did not continuously follow post-exposure PRR-expression over time. However, in agreement with the low basal expression of TLR2 on monocytes, farmers had lower TLR2 expression on monocytes than controls after *ex vivo* stimulation with dust. In the present study, the differences between the groups are mostly TLR2 specific, implying that the attenuated inflammatory reaction observed in farmers compared to unexposed healthy subjects after exposure in a pig barn[11] might be caused by tolerance against TLR2 ligands (predominantly from Gram-positive bacteria) and not LPS.

In contrast to our results, Lauener *et al*.[26] have shown that children growing up on a farm, implying that they are continuously exposed to PAMPs, have increased gene

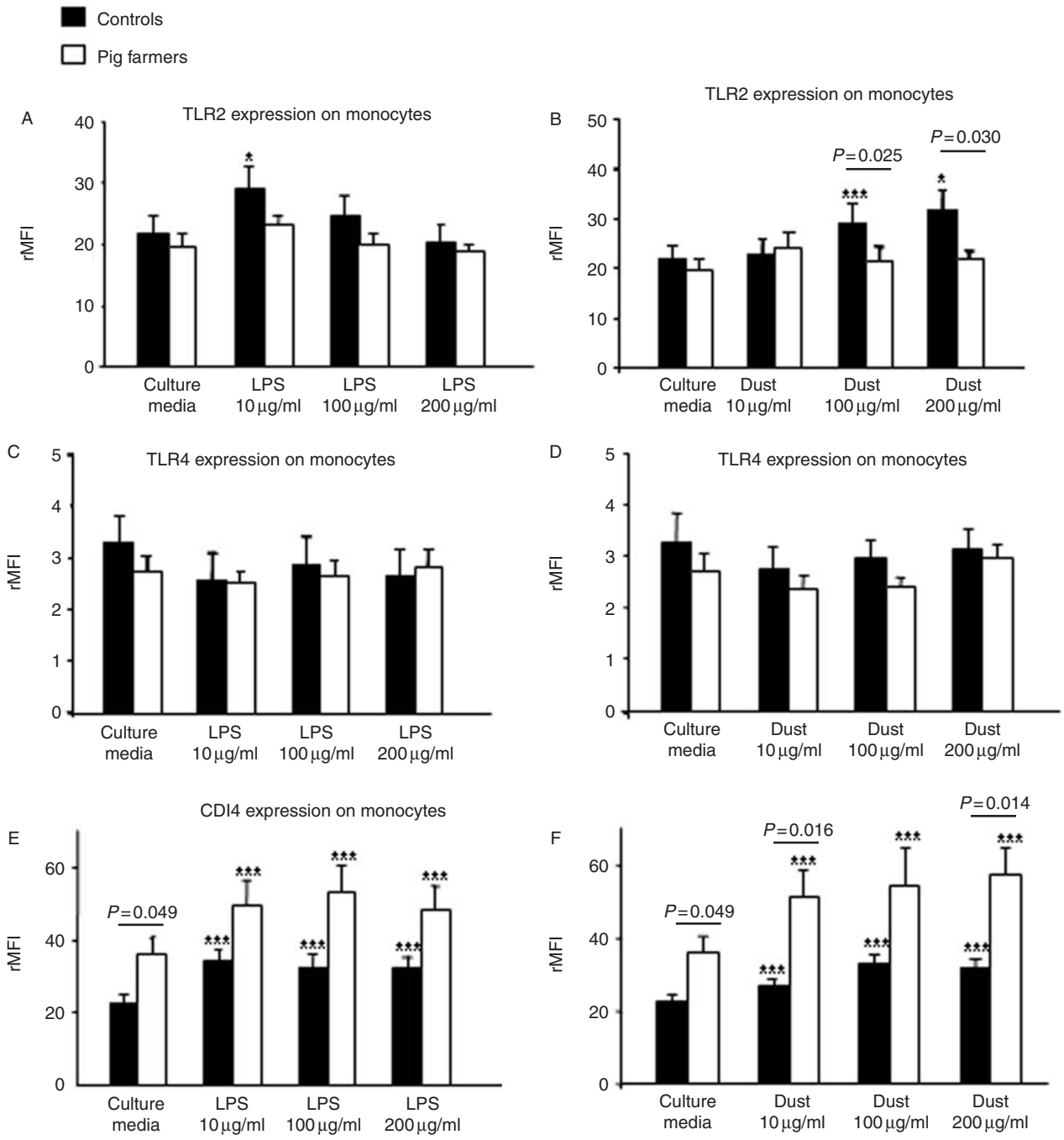

Fig. 7. Surface expression of TLR2 (A,B), TLR4 (C,D) and CD14 (E,F) on peripheral blood monocytes after *ex vivo* stimulation with LPS (A,C,E) or dust collected in a pig barn (B,D,F). ($n = 10$–12). $^{*}P < 0.05$, $^{***}P < 0.001$, compared with media. Brackets indicate differences between the groups. rMFI = relative mean fluorescence intensity.

expression of TLR2 and CD14. It thus seems reasonable to conclude that daily heavy exposure to the pig barn environment down-regulates TLR2 expression on circulating monocytes while the opposite seems to be the case when exposure is substantially lower and earlier in life. However, the present study presents cell surface expression of TLR2 protein in pig farmers and does not give definite evidence that TLR2 is decreased on gene level.

Interestingly, LeBouder *et al.*[27] have shown that monocytes, possibly as a negative feed back, release soluble TLR2 (sTLR2) in response to cell activation and that the cellular response is increased after sTLR2 depletion. The decreased TLR2 expression on peripheral blood monocytes (assessed before exposure) might be due to enhanced release of sTLR2 as a result of the intense exposure in the pig barn environment.

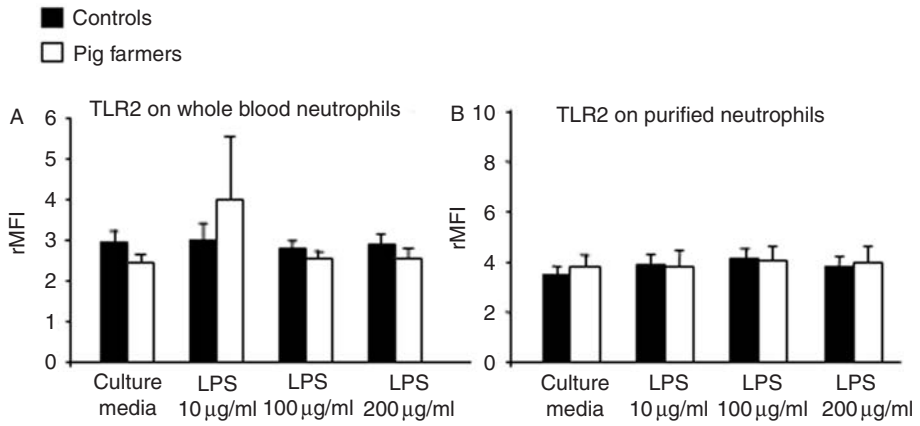

Fig. 8. Surface expression of TLR2 on peripheral blood neutrophils (A) and purified neutrophils (B) after LPS stimulation, *ex vivo*. rMFI = relative mean fluorescence intensity ($n = 10–12$).

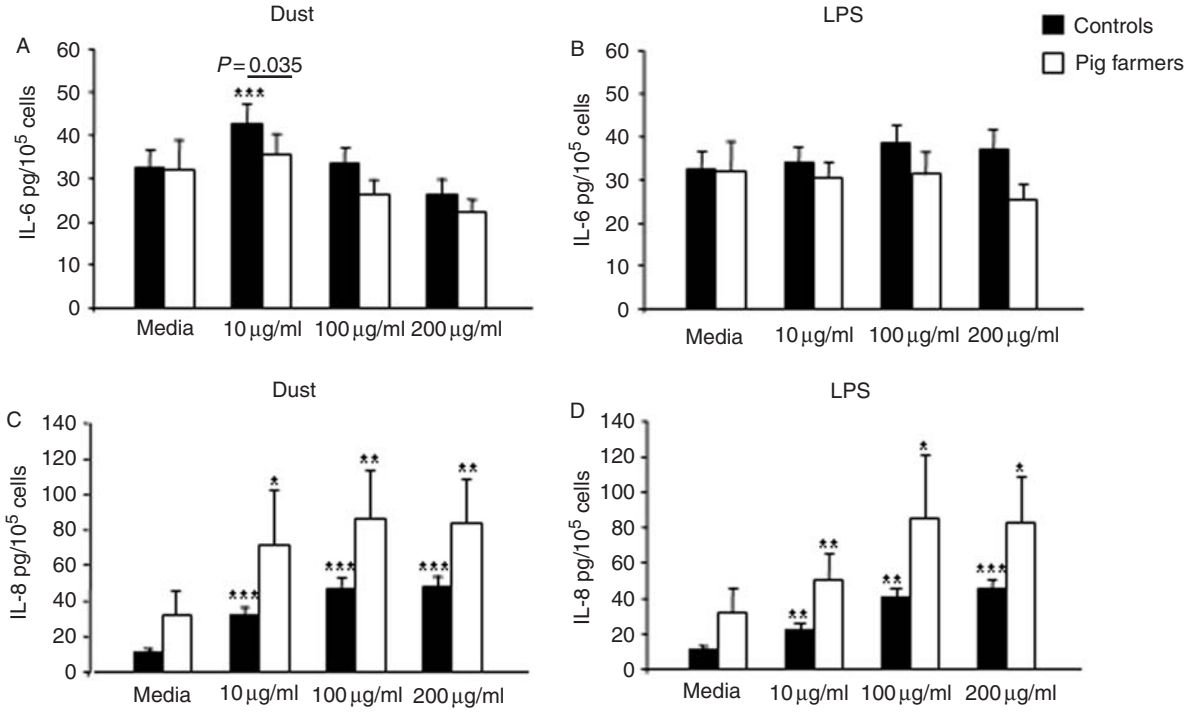

Fig. 9. Release of IL-6 and IL-8 after LPS and dust stimulations *ex vivo* of peripheral blood. ($n = 10–12$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with media. Brackets indicate differences between the groups.

Further, sTLR2 is known to suppress release of pro-inflammatory cytokines.[27] This might explain the attenuated response of IL-6 in serum in farmers after exposure in a pig house compared to controls, as also has been previously shown.[11] Since development of tolerance also has been described following repeated exposure to LPS[9,10] and it has been shown that smoke from cigarettes contains high levels of bioactive LPS,[15,16] the findings that smokers neutrophil numbers and IL-6 levels in serum increased significantly more after LPS provocation were surprising. This is, however, in line with the findings by Wesselius *et al*.,[28] who showed that both neutrophil numbers and IL-1β increased significantly more in smokers than non-smokers in BAL-fluid after LPS inhalation.

Apart from sTLR2 there are several other identified negative regulators of the TLR intracellular signalling pathway,[10,29,30] such as A20 (de-ubiquitylates tumour-necrosis factor-receptor-associated factor 6 [TRAF6])[31,32] and the IκBα-stabilizing TNF-related apoptosis-inducing ligand receptor (TRAIL-R)[33] that may be involved in the tolerogenic phenomenon observed in pig farmers. The increased proportion of Th cells of the Th2 type observed in the present study

may also influence TLR2 expression as it has been shown that both IL-13 and IL-4 have the ability to down-regulate PRR gene and surface expression on human intestinal epithelial cells and U937 monocytic cell line.[34]

Interleukin-13 and IL-4 are involved in the pathogenesis of allergy and asthma[35] but are also known as anti-inflammatory cytokines, with a protective effect on lethal endotoxemia in animal experiments.[36,37] These cytokines are likely to be important in suppressing the acute inflammation after exposure to pig dust and LPS challenge. Thus, these cytokines may exhibit a counter regulation to exposure and, thereby, contribute to the finding of enhanced proportion of IL-13 and IL-4 producing cells in farmers and smokers compared to controls. As IL-4 and IL-13 producing Th were high at baseline there was no space for further increase in the chronic exposed groups as was the case in the controls. The increased proportion of IL-13 and IL-4 producing Th cells in pig farmers and smokers thus indicates that those cytokines are involved in chronic inflammation. The positive correlation between the increased proportion of IL-13 producing Th cells and the total cumulative exposure to pig house environment in the farmers also supports this claim.

The perpetual increase of IL-13 producing Th2 cells may induce negative tissue effects as IL-13 and IL-4 are involved in the pathogenesis of a number of inflammatory disorders characterised by tissue remodelling.[19,38,39] Recently, it was shown that IL-13 signalling trough the IL-13α2 receptor induces production of transforming growth factor (TGF)-β_1, a growth factor known to be involved in pulmonary fibrosis[40] and most likely contributes to the peribronchiolar fibrosis observed in COPD.[41] Thereby, the increased proportion of IL-4 and IL-13 producing Th cells, observed in farmers and smokers in the present study, is likely to reflect an ongoing chronic airway inflammation. If this activity is related to later development of COPD observed among farmers[13] is not known.

Conclusions

Pig farmers and smokers, who are continuously exposed to organic material and microbial compounds, have an increased number of inflammatory cells in peripheral blood and an increased proportion of circulating Th2-type lymphocytes which, among pig farmers, were positively related with the duration of work in pig confinement buildings. In addition, pig farmers have lower expression of TLR2 on peripheral blood monocytes than have smokers and controls. Compared to the non-farming control group, farmers showed an attenuation of mainly TLR2 expression after acute exposure and *ex vivo* stimulations, implying activation of innate immune receptors already at baseline (pre-exposure). The altered innate immune response may in the long term have negative effects on the tissue and, thereby, be involved in the pathogenesis of respiratory disorders such as chronic bronchitis, which frequently occurs among smokers and pig farmers.

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