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Lymphocyte activation after exposure to swine dust: a role of humoral mediators and phagocytic cells

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Lymphocyte activation after exposure to swine dust: a role of humoral mediators and phagocytic cells. C. Müller-Suur, P.H. Larsson, K. Larsson, J. Grunewald. ©ERS Journals Ltd 2002.

ABSTRACT: Exposure to swine dust causes intense airway inflammation with multifold increase in inflammatory cells and secretion of pro-inflammatory cytokines. This *in vitro* study focuses on the swine-dust activation of lymphocytes in whole blood, in phagocyte-depleted whole blood and in peripheral blood mononuclear cells (PBMC), in order to investigate whether phagocytic cells and/or soluble mediators are involved in the activation of T-cells following exposure to organic dust from a swine confinement house.

T-cell activation was analysed by flow cytometry with double staining for CD3 and the activation marker CD69.

Swine dust (50 µg) incubated (24 h) with heparinized whole blood was shown to activate 27.6% of the T-cells, while swine dust incubated with whole blood depleted from phagocytic cells or PBMC only activated 4.5% and 4.8% of the T-cells, respectively. Plasma separated from whole blood preincubated with swine dust for 24 h stimulated as much as 32.4% of PBMC T-cells and contained high levels of interleukin (IL)-12 (14 pg·mL) and interferon (IFN)-γ (2284 pg·mL^{-1}), while plasma from PBMC incubated with swine dust contained low levels of IL-12 (2 pg·mL^{-1}) and IFN-γ (196 pg·mL^{-1}).

This study demonstrates that activation of T-cells by organic dust from a swine confinement building seems to require phagocytic cells, most likely acting through the release of soluble mediators. Also, conditioned plasma from swine-dust exposed whole blood, which was capable of activating T-cells, contained high concentrations of interleukin-12 and interferon-γ.

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Three hours exposure to dust in a swine confinement building causes intense airway inflammation, with a multifold increase of inflammatory cells, predominantly neutrophils, in healthy volunteers [1]. The exposure also induces secretion of pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-8 in the airways *in vivo* as assessed by bronchoalveolar lavage (BAL) [2–4]. The most probable sources for these cytokines are alveolar macrophages [5] and epithelial cells [6]. Previous *in vivo* findings have shown increased numbers of T- and B- lymphocytes in BAL fluid following exposure to dust in a swine confinement house, but the proportion of T-lymphocytes in relation to the total amount of lymphocytes, was unaltered [7]. In the same study it was also demonstrated that exposure to organic dust induced activation (increased expression of the activation markers CD69, CD25 and human leukocyte antigen-DR) of T-lymphocytes *in vivo* [7]. Furthermore, dust from swine confinement buildings activates T-cells in a dose- and time-response manner *in vitro* when incubated with peripheral whole blood from healthy subjects [8].

The *in vivo* lymphocyte activation caused by inhaled organic dust may comprise a specific activation of the T-lymphocytes through the T-cell antigen receptor. There is also a possibility that the activation of T-cells is mediated by nonspecific mechanisms in which release of T-cell activating soluble mediators from cells other than lymphocytes may be involved.

The aim of the present *in vitro* study thus was to elucidate whether phagocytic cells and/or soluble mediators are involved in the activation of T-cells following exposure to organic dust from a swine confinement house.

Materials and methods

Subjects

Venous blood was collected from seven (5 female), nonallergic, nonsmoking, healthy subjects, with no previous exposure for swine dust, and with a mean age of 43 (range 26–58) yrs. All subjects gave their informed consent and the study was approved by the Ethics Committee of Karolinska Institute, Stockholm, Sweden.

Stimulation with dust

Dust collected ~1.5 m above the floor in a swine confinement building, was dissolved in Roswell Park

Memorial Institute (RPMI) 1640 medium (Gibco Laboratories, Paisley, UK), sonicated for 10 min and diluted in heparinized whole blood to a final concentration of 50 $\mu g \cdot mL^{-1}$. The polyclonal activator, phytohaemagglutinin (PHA), used as positive control, was also dissolved in RPMI 1640 and sonicated for 10 min and diluted in whole blood to a final concentration of 10 $\mu g \cdot mL^{-1}$. Unstimulated whole blood containing RPMI 1640, was used as negative control. Blood samples were incubated for 24 h and then ethylenedianmine tetracetic acid (EDTA) was added to a final concentration of 5 mM. All stimulation tests were performed in duplicate during 24 h in 37°C and 5% CO_2.

Blood was collected using Vacutainer® brand blood collection tubes, and Vacutainer® CPT™, (Cell Preparation Tubes, Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) containing sodium heparin anticoagulant. Peripheral blood mononuclear cells (PBMC) were selected through gradient separation. In a first experimental set-up PBMC was incubated with plasma received from whole blood previously incubated with 50 $\mu g \cdot mL^{-1}$ swine dust, *i.e.* "conditioned plasma", 10 $\mu g \cdot mL^{-1}$ PHA or RPMI for 24 h. In a second experimental set-up, PBMC was resuspended in autologous plasma and incubated with 50 $\mu g \cdot mL^{-1}$ swine dust, 10 $\mu g \cdot mL^{-1}$ PHA or RPMI for 24 h.

Phagocytic cells were removed from whole blood through incubation with carbonyl iron particles (4 $\mu g \cdot mL^{-1}$, size 5 µg, Sigma Aldrich, Stockholm, Sweden). Blood and carbonyl iron particles were incubated for 30 min at 37°C and mixed occasionally. After incubation, the blood sample tube was placed on one of the pools of a magnet (Dynal A.S., Oslo, Norway) for 10 min at 4°C. The noniron containing *i.e.* the nonphagocytic cells were removed (with the tube still standing on the magnet) and transferred to a second plastic tube. Cells were resettled on the magnet for a further 10 min at 4°C and the procedure was repeated. Blood was stained with the monoclonal antibody CD14-PE/CD45-FITC (Becton Dickinson, Franklin Lakes, NJ, USA) before and after iron incubation. The nonphagocytic cells were incubated with 50 µg swine dust·mL^{-1}, 10 µg PHA·mL^{-1} or with RPMI 1640 (negative control) for 24 h.

Monoclonal antibodies and flow cytometric analysis

Cells were characterized by subgroup-specific fluorochrome-conjugated monoclonal antibodies. For analysis of T-cell activation, double staining for CD3 and the cell-surface activation marker CD69 (Becton Dickinson) was performed. Monocyte depletion was evaluated and analysed by CD14-PE/CD45-FITC. Twenty microlitres of monoclonal antibody was added to 100 µL aliquots of either blood or PBMC samples dissolved in plasma. The samples were incubated 20 min in the dark, at room temperature. Lysing of red blood cells, was done using fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson) and fixation was achieved by using Cellfix (Becton Dickinson). For background staining, isotypic controls were performed. Cell count percentages were calculated by flow cytometry using a FACS Calibur (Becton Dickinson).

Cytokine analyses by enzyme-linked immunosorbent assay

IL-12 in plasma samples was analysed by Human IL-12 immunoassay (R&D System Europe Ltd, Abingdon, UK). Interferon (IFN)-γ in plasma samples was analysed by OptEIA™ Human IFN-γ Kit (PharMingen, San Diego, CA, USA).

Statistics

Statistical analysis was performed using StatView® programme, version 4.02 for Macintosh and Super Anova 1.11 (Abacus Concepts, Inc, Berkeley, CA, USA). Results are presented as medians (25–75th percentiles). Comparisons were performed by analysis of variance (ANOVA). P-values <0.05 were considered to be statistically significant.

Results

After 24-h incubation of whole blood *in vitro* 27.6% (17.3–30.8) of the CD3 positive lymphocytes (T-cells) expressed the early activation marker CD69 compared to 3.3% (2.2–4.4) of T-lymphocytes in nonstimulated whole blood ($p<0.0001$). Incubation with PHA and whole blood yielded 71.3% (65.3–73.4) of the T-lymphocytes to express CD69 (fig. 1).

In PBMC selected from whole blood and incubated with dust for 24 h CD69 was expressed in 4.8% (4.5–7.0) of the T-cells compared to 2.2% (1.5–3.1) of T-lymphocytes from nonstimulated PBMC (not significant). PHA induced CD69 expression in 70.9% (36.0–78.8) of the T-lymphocytes in PBMC (fig. 1).

Phagocytic cells were removed from whole blood through incubation with carbonyl iron, reducing the proportion of monocytes from 7 to 1% and of granulocytes from 58 to 35% (median value). In such "phagocyte reduced" whole blood, incubation with dust for 24 h induced CD69 expression in 4.5% (3.6–9.7) of the T-lymphocytes as compared to 1.5% (1.2–1.7) in T-lymphocytes of nonstimulated "phagocyte reduced" whole blood (not significant). Incubation with PHA as a positive control induced CD69 expression on 71.3% (59.1–79.2) of the T-lymphocytes (fig. 1).

Plasma was transferred from dust-exposed whole blood, *i.e.* "conditioned plasma", and incubated with PBMC for 24 h. Following this incubation, 32.4% (29.2-34.4) of the CD3 positive T-lymphocytes expressed CD69 compared to 3.3% (2.8–3.6) of T-lymphocytes of PBMC incubated with control plasma ($p<0.0001$). Plasma from whole blood stimulated with PHA induced expression of CD69 in 37.1% (30.0–52.1) of the T- lymphocytes (fig. 2).

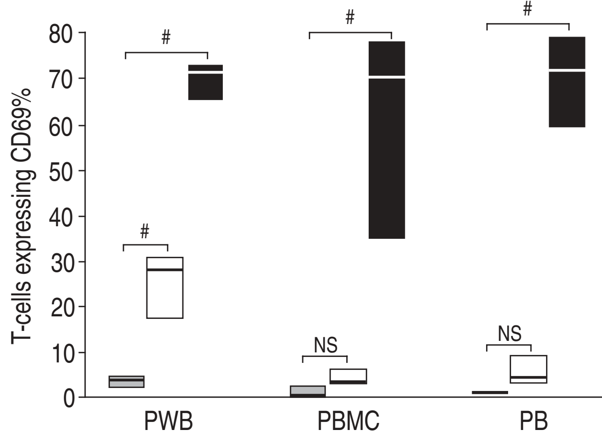

Fig. 1. – CD69 expression of T-lymphocytes (% of CD3 positive cells) in peripheral whole blood (PWB), peripheral blood mononuclear cells (PBMC) resuspended in autologous plasma and peripheral blood depleted from phagocytic cells (PB), following incubation with either Roswell Park Memorial Institute medium (control: ■), swine dust (□) or phytohaemoglutin (■) for 24 h. Values are presented as median values and interquartile ranges. NS: no significant difference; #: $p<0.0001$ compared to unstimulated cells.

Soluble mediators in conditioned plasma

In an attempt to identify soluble mediators of importance for the T-lymphocyte stimulation, the concentration of IL-12 and IFN-γ in conditioned plasma was measured. In the dust incubated whole blood, *i.e.* the conditioned plasma, the IL-12 concentration was 14.1 (8.3–22.1) $pg \cdot mL^{-1}$, while the concentration was below detection limit in control plasma. Plasma from PHA stimulated whole blood contained 47.4 (25.7–73.2) $pg \cdot mL^{-1}$ IL-12. In plasma of PBMC stimulated with dust, the IL-12 concentration was 2.3 (0.1–2.8) $pg \cdot mL^{-1}$. PHA stimulated PBMC supernatant contained 5.9 (3.5–10.6) $pg \cdot mL^{-1}$, while negative controls had undetectable levels (fig. 3).

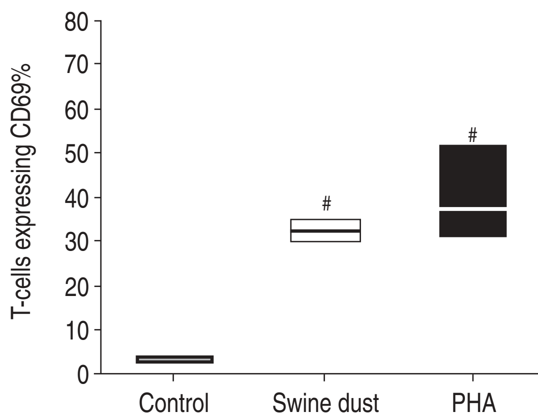

Fig. 2. – CD69 expression of T-lymphocytes (% of CD3 positive cells) in peripheral blood mononuclear cells (PBMC) following 24 h incubation with Roswell Park Memorial Institute medium (RPMI: control), plasma from swine dust (white box) or phytohaemoglutin (PHA: black box) stimulated whole blood. Values are presented as median values and interquartile ranges. #: $p<0.0001$ compared to control.

IFN-γ levels in conditioned plasma were 2284.1 (1352.8–4025.9) $pg \cdot mL^{-1}$ compared to 4775.8 (3936.2–9381.8) $pg \cdot mL^{-1}$ in PHA stimulated plasma and 6.5 (0.7–32.6) $pg \cdot mL^{-1}$ in control plasma. PBMC plasma from swine dust incubations contained 195.9 (161.2–364.9) $pg \cdot mL^{-1}$ compared to 804.1 (286.7–863.0) $pg \cdot mL^{-1}$ in PHA stimulated PBMC and 6.0 (1.9–15.0) $pg \cdot mL^{-1}$ in control supernatant (fig. 3).

Discussion

In line with the authors' previous findings [8] the present study demonstrates that swine dust activates T-lymphocytes in whole blood *in vitro*, as detected by T-cell expression of the early activation marker CD69. However, when incubating swine dust with PBMC separated from whole blood, T-lymphocytes were not activated. In contrast to whole blood, PBMC consists mainly of lymphocytes, while there are virtually no granulocytes and only few monocytes. To further investigate whether phagocytic cells such as granulocytes and monocytes could influence the dust-mediated activation of T-lymphocytes, the number of monocytes and granulocytes in whole blood were reduced, which substantially reduced the capacity of the dust to activate T-lymphocytes. Organic dust obtained from swine confinement houses is therefore capable of activating T-lymphocytes only in the presence of phagocytic cells. Moreover, plasma from swine dust exposed whole blood, *i.e.* conditioned plasma induced lymphocyte activation. Based on these data the present authors conclude that T-cell activation induced by dust requires phagocytic cells and that it is induced by mediator release from phagocytic rather than by a direct cell-to-cell interaction.

The present authors previously found dramatically increased numbers of inflammatory cells, especially neutrophilic granulocytes, in BAL fluid after exposure to swine dust *in vivo*. Increased concentrations of TNF-α, IL -1, IL-6 and IL-8 were also found in BAL fluid following exposure to swine dust *in vivo* [3, 4] and in supernatant of epithelial cells and alveolar macrophages following exposure to dust *in vitro* [9, 10]. Granulocytes have also been shown to release many of these cytokines, *i.e.* TNF-α, IL -1, and IL-8 upon lipopolysaccharide (LPS) stimulation [11–15], and monocytes can produce TNF-α [16], IL-1, [15, 17, 18] and IL-12 [19]. Many of these granulocytes and monocytes derived cytokines are involved in the activation of lymphocytes, either directly (IL-1, IL-6, IL-12) or indirectly (IFN-γ). In the present study, high levels of IL-12 and IFN-γ were found in conditioned plasma following *in vitro* exposure of whole blood to dust. Thus phagocytic cells such as granulocytes and monocytes present in whole blood during swine dust incubation seem to release cytokines IL-12 and IFN-γ which may be of importance for the swine dust induced T-lymphocyte stimulation. It seems likely that monocytes produce cytokines such as IL-12, which may induce IFN-γ production by lymphocytes such as natural killer (NK) cells.

To conclude, phagocytic cells are necessary for the dust-activation of T-lymphocytes. The phagocytes

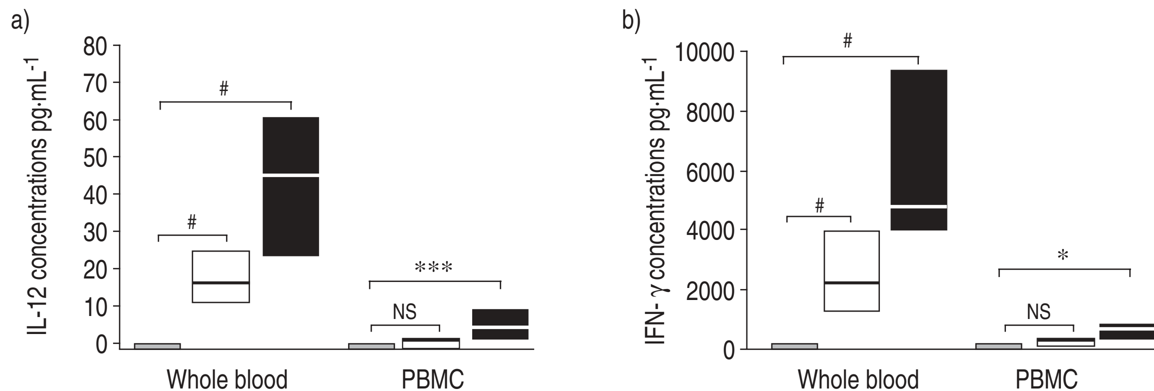

Fig. 3. – Interleukin (IL)-12 and interferon (IFN)-γ concentrations in plasma from whole peripheral blood and peripheral blood mononuclear cells (PBMC), incubated with either Roswell Park Memorial Institute medium (RPMI) (control: ■), swine dust (□) or phytohaemoglutin (■) for 24 h. Values are presented as median values and interquartile ranges. NS: no significant difference; *: $p<0.05$; ***: $p<0.001$; #: $p<0.0001$ compared to unstimulated cells.

seem to act by releasing lymphocyte-activating cytokines such as interleukin-12 and interferon-γ. To improve the understanding of immune response following exposure to organic dust, intracellular cytokine release from swine-dust activated peripheral blood cells as well as bronchoalveolar lavage cells from dust-exposed individuals will need to be studied *in vivo*.

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