

## Ambient air particles from four European cities increase the primary cellular response to allergen in the draining lymph node

Unni Cecilie Nygaard<sup>a,\*</sup>, Torunn Alberg<sup>a</sup>, Rob Bleumink<sup>b</sup>, Audun Aase<sup>c</sup>,  
Erik Dybing<sup>a</sup>, Raymond Pieters<sup>b</sup>, Martinus Løvik<sup>a</sup>

<sup>a</sup> Division of Environmental Medicine, Norwegian Institute of Public Health, P.O. Box 4404 Nydalen, NO-0403 Oslo, Norway

<sup>b</sup> Institute for Risk Assessment Sciences (IRAS), Immunotoxicology, Utrecht University, Yalelaan 2, 3584 CM Utrecht, The Netherlands

<sup>c</sup> Division of Infectious Disease Control, Norwegian Institute of Public Health, P.O. Box 4404 Nydalen, N-0403 Oslo, Norway

Received 8 July 2004; received in revised form 14 September 2004; accepted 14 September 2004

Available online 14 November 2004

### Abstract

In the RAIAP (respiratory allergy and inflammation due to ambient particles) project, qualitative properties of ambient air particles from Amsterdam, Oslo, Lodz and Rome were investigated in relation to inflammation and allergy. Most collected particle fractions were found to increase the allergen-specific IgE and IgG2a responses after subcutaneous injection of particles with allergen in mice. However, some fractions appeared to skew the antibody response towards more Th1- or Th2-associated antibody isotypes, and the fine fractions were found to be more potent than the coarse fractions with regard to IgE adjuvant activity. In the present study we investigated the cellular response in the draining lymph node 5 days after a subcutaneous injection of selected RAIAP particle fractions. The particles (100 µg) were injected into both hind footpads of BALB/cA mice, in the presence or absence of the allergen ovalbumin (OVA, 50 µg). We also studied if the coarse and fine RAIAP particle fractions affected the cellular responses to OVA differently. The number of lymph node cells, as well as the relative number of B and T lymphocytes and T helper cells were determined. Expression of cell surface molecules (MHC class II, CD86 and CD23) and ex vivo cytokine production (IL-4, IL-10 and IFN-γ) by the lymph node cells were measured. Overall, particles in the presence of allergen enhanced the levels of the various cellular parameters compared to allergen alone or particles alone. In the absence of allergen, ambient air particles, in contrast to diesel exhaust particles, marginally affected some cellular parameters. By histological examination of the lymph node, the particles appeared to be scattered between the lymphocytes, often localised

\* Corresponding author. Tel.: +47 2204 2257; fax: +47 2204 2686.

E-mail address: [unni.cecilie.nygaard@fhi.no](mailto:unni.cecilie.nygaard@fhi.no) (U.C. Nygaard).

within macrophage-like (acid phosphatase positive) cells. The cell parameters measured could, for the individual sample, neither predict the degree of a Th2- or Th1-skewed antibody response, nor the stronger antibody adjuvant capacity of the fine than the coarse particle fractions. In conclusion, we have shown that coarse and fine ambient air particles from different European cities enhance the cellular response in the draining lymph node after injection with an allergen. In the absence of allergen, ambient particles only marginally affected the cellular parameters.

© 2004 Elsevier Ireland Ltd. All rights reserved.

**Keywords:** Air pollution; Particles; Adjuvant; Allergy; Cytokines; Cell surface molecules

## 1. Introduction

In Europe, the prevalence of asthma and allergic diseases has been reported to be higher in the western than the eastern countries (von Mutius et al., 1992, 1994; Jogi et al., 1998; ISAAC, 1998). Among several factors characterising the “western life-style”, traffic-related ambient air particles have been suggested to play a role in inducing and worsening asthma and allergic diseases (Parnia et al., 2002). Particulate air pollution has differed between eastern and western European countries, the eastern have been dominated by coarse particles and the western by fine particles largely derived from combustion engines (Schafer and Ring, 1997; Ebelt et al., 2001; Williams, 1999). Further, epidemiological studies have indicated regional and seasonal differences in the pooled estimated risks for short-term health effects of ambient air particles (Katsouyanni et al., 2001; Atkinson et al., 2001). Therefore, to investigate whether qualitative differences in particulate air pollution at different European locations in part may explain differences in prevalence or severity of respiratory allergies and asthma throughout Europe, the RAIAP project was conducted (Dybing et al., 2004). Coarse (2.5–10  $\mu\text{m}$ ) and fine (0.1–2.5  $\mu\text{m}$ ) particle fractions were collected in four European cities (Amsterdam, Oslo, Lodz, and Rome) during three seasons (the spring, summer and winter of 2001/2002; Cassee et al., 2003). The particle fractions have been characterised for physical, chemical and immunological parameters. Furthermore, the allergic adjuvant capacity of the collected particles was recently determined after subcutaneous injection of particles with the allergen ovalbumin (OVA) into the footpad of mice (Lovdal et al., 2003), employing the model described in Lovik et al. (1997). Most collected particle fractions, except for a few of the coarse ones, increased both the allergen-specific IgE (Th2-associated) and IgG2a

(Th1-associated) antibody levels in serum. However, some samples appeared to skew the antibody response towards more Th1- or Th2-associated antibody isotypes. Overall, the fine fractions were found to be more potent than the coarse fractions with regard to IgE adjuvant activity.

The underlying mechanisms for the IgE adjuvant effect of ambient air particles are not known. However, both the particle core and particle-associated substances have been shown to contribute to the effect of particles on the immune system (reviewed in Granum and Lovik, 2002). Further, widely different particle types have been shown to enhance either Th1- or Th2-associated, or mixed, responses (van Zijverden et al., 2000; Maejima et al., 1997; Granum et al., 2001a). Moreover, we have recently shown that particle size is an important determinant of the adjuvant effect of polystyrene particles (PSP), as fine PSP (0.0588 and 0.202  $\mu\text{m}$  diameter) had much higher adjuvant capacity than coarse PSP (1.053, 4.64 and 11.14  $\mu\text{m}$ ) in the subcutaneous injection model (Nygaard et al., *in press*). The primary immune response to an antigen mainly takes place in the draining lymph nodes (Zinkernagel, 2000), where IgE antibody-producing cells have been found (Sedgwick and Holt, 1985; Gutting et al., 2002; van Zijverden et al., 2000). We therefore wanted to study how the particles, alone or together with an allergen, affected the immune cells in the draining lymph node during the primary immune response. We have earlier shown that the IgE adjuvant effect of PSP (0.1  $\mu\text{m}$  diameter) was associated with increased primary cellular responses in the popliteal lymph node (PLN), peaking 5 days after subcutaneous injection into the footpad (Nygaard et al., 2004). In the present study we tested whether coarse and fine size fractions of ambient air particulate matter from different European cities alter the allergic immune response by modulating the expression of cell membrane molecules and the

pattern of cytokine production in the draining lymph node cells. We examined a selection of RAIAP particle fractions which appeared to exert different adjuvant effects with regard to Th1- or Th2-associated antibody responses (Lovdal et al., 2003). In addition, we investigated if any of the measured cellular parameters were more influenced by the fine than the coarse particle fractions. A further aim was to investigate whether differences in cellular responses in the draining lymph node could predict differences in antibody adjuvant activity.

## 2. Materials and methods

### 2.1. Animals

Female inbred BALB/cA mice (Taconic M&B A/S, Ry, Denmark) were housed, 5 or 4 animals per cage, on BeeKay bedding (B&K Universal AS, Nittedal, Norway) in type III macrolon cages in filter cabinets (Scantainers). The mice were exposed to a 12 h/12 h light/dark cycle (30–60 lx in cages), room temperature ( $20 \pm 2^\circ\text{C}$ ) and 40–60% relative humidity. The animals were given pelleted food (RM1, SDS, Essex, UK) and tap water ad libitum. All animals were allowed to rest for at least 1 week before entering the experiments 6–7 weeks old. The experiments were performed in conformity with the laws and regulations for experiments with live animals in Norway, and they were approved by the Experimental Animal Board under the Ministry of Agriculture in Norway.

### 2.2. Particles

Ambient air particles were collected within the RAIAP framework, in Oslo, Rome, Lodz, and Amsterdam during the spring, summer, and winter of 2001/2002. Coarse (2.5–10  $\mu\text{m}$ ) and fine (0.1–2.5  $\mu\text{m}$ ) particle fractions were extracted with methanol from the polyurethane foams of the high-volume sampler, evaporated and dried (Cassee et al., 2003). Diesel Particulate Matter (DEP; Standard Reference Material 1650a, Institute of Standards & Technology, Gaithersburg, MD, USA), which according to the manufacturer consists of mainly fine particles, was included as a positive reference particle. The adjuvant capacity of all RAIAP particle fractions on allergen-specific an-

tibody responses were determined after subcutaneous injection of particles with OVA (Lovdal et al., 2003). Based on the antibody responses, two sets of particles with different adjuvant capacities were selected. The first set of particles consisted of Diesel Exhaust Particles (DEP), the fine particle fraction from Rome (spring) and the fine particle fraction from Oslo (summer, in the following called Oslo1). Compared to OVA alone, these particles increased the Th2, Th2 > Th1 and Th1 > Th2 associated antibody responses, respectively. The second set of particles consisted of coarse and fine particle fractions from Amsterdam (spring), Lodz (summer) and Oslo (spring, in the following called Oslo2). These fine fractions appeared to induce higher antibody levels than their coarse counterparts.

### 2.3. Immunisation with allergen and particles

The allergen ovalbumin (OVA, Gal d2; chicken egg albumin, grade VII, Sigma, St. Louis, MO, USA) was given at an amount of 50  $\mu\text{g}$  per injection, whereas 100  $\mu\text{g}$  of particles were given per injection. The test suspensions were injected into both hind footpads to reduce the number of animals used, but still obtain a sufficient number of popliteal lymph node cells. Particles were suspended in Hank's balanced salt solution (HBSS; PAA Laboratories GmbH, Linz, Austria) with or without OVA, and stirred at  $18^\circ\text{C}$  over night using a magnetic stirrer. For the lymph node histology, also 40  $\mu\text{g}$  of fluorescent polystyrene particles (PSP, 0.1  $\mu\text{m}$  diameter; Fluoresbrite™ Yellow Green (YG) Microspheres, Polysciences Europe GmbH, Eppenheim, Germany) were injected with and without 50  $\mu\text{g}$  OVA. The PSP suspensions were prepared on the day of injection. For all solutions, 20  $\mu\text{l}$  were injected subcutaneously into both hind footpads (heel-toe direction) using a 100  $\mu\text{l}$  Hamilton syringe (Hamilton Bonaduz AG, Switzerland) with a 30 G needle (BD Medical Systems, Ireland).

### 2.4. Lymph node cell preparation

Five days after injection, the animals were killed by cervical dislocation under  $\text{CO}_2$  anaesthesia, and both popliteal lymph nodes (PLN) were excised and kept on ice in HBSS with 2% foetal calf serum (FCS; GibcoBRL, Auckland, New Zealand). Single cell sus-

pensions were prepared from each lymph node as previously described (Nygaard et al., 2004). After pooling of the two PLN cell suspensions from the same animal, the cell concentration was measured with a Coulter counter Z1 (Beckman Coulter Incorporated, FL, USA), and presented as the lymph node cell number ( $10^6$ ) per PLN.

### 2.5. Cell phenotyping

PLN single cell suspensions were stained as previously described (Nygaard et al., 2004). In short,  $5 \times 10^6$  cells/ml were stained with FITC- or PE-conjugated monoclonal antibodies against CD19 (B lymphocyte marker), CD4 (T helper cell marker), CD86, CD23 (BD PharMingen, San Diego, CA, USA) or CD3 $\epsilon$  (T lymphocyte marker), MHC class II (Southern Biotech, Birmingham, AL, USA). Positive cells were generally defined as the cell population with fluorescence intensity stronger than 99% of the isotype control-stained cells. A minimum of 10,000 lymphocytes were analysed on an EPICS XL instrument (Coulter, FL, USA) connected to a PC using EXPO32™ Software (Applied Cytometry Systems, Sheffield, UK). Expression of surface molecules was determined in two ways: (1) the relative number of positive cells and (2) the relative amount of epitopes per cell measured as median fluorescence intensity. After double staining for the specific marker and anti-CD19, the results are presented as the expression of the specific marker per B lymphocyte.

### 2.6. Ex vivo cytokine production and detection by ELISA

Ex vivo cytokine production from PLN single cell suspensions was determined as previously described (Nygaard et al., 2004). In short, a pool of cells from three animals was resuspended in culture medium, and  $5 \times 10^6$  cells were cultured for 24 and 48 h with  $5 \mu\text{g/ml}$  of the mitogen Concanavalin A (ConA, Sigma, MO, USA). The cell supernatant levels of IL-4, IL-10 and IFN- $\gamma$  were determined by sandwich ELISA according to the manufacturer's protocol (Mouse DuoSets, R&D Systems Inc., MN, USA). The IL-4, IL-10 and IFN- $\gamma$  levels after the optimal culture times 24, 24 and 48 h, respectively, are presented.

### 2.7. Lymph node histology

PLN from both legs were excised and frozen in Tissue-Tek O.C.T. compound (Miles Inc., IN, USA), and  $7 \mu\text{m}$  cryostat sections were fixed in acetone. Sections were incubated for 1 h at room temperature with a mixture of rat anti-mouse B220 (B cell marker, clone B220) and hamster anti-mouse CD3 (T cell marker, clone 145-2c11) monoclonal antibodies in phosphate buffer (PBS, pH 7.2) with 1% bovine serum albumin (BSA). After three washes in PBS/Tween, sections were incubated in a mixture of biotin-conjugated goat anti-hamster immunoglobulins (1:100; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) and peroxidase-conjugated rabbit anti-rat (1:40, DAKO, Denmark), for 1 h at room temperature. Followed by three washes, the sections were further incubated with alkaline phosphatase-labelled streptavidin (1:800; Boehringer Mannheim GmbH, Mannheim, Germany). After subsequent washes in PBS/Tween and acetate buffer (200 mM, pH 5.4), macrophages were identified by acid phosphatase staining using naphthol ASBI phosphate and red-violet LB salt in acetate buffer for 30 min at  $37^\circ\text{C}$ . B cells were visualised by peroxidase-based staining with diaminobenzidine, whereas the T cells were visualised by NBT/BCIP (nitro blue tetrazolium/bromo-chloro-indoryl phosphate). Separate slides were also stained for macrophages (as described above), and thereafter counter-stained with Mayer's acid haematoxylin. The sections were covered with an aqueous mounting medium. The PLN sections containing fluorescent PSP were covered without any treatment, and analysed by fluorescence microscopy.

### 2.8. Statistical analysis

Statistical analysis was performed with SigmaStat® Statistical Analysis System for Windows Version 2.03 (Jandel Scientific, Erkrath, Germany). For each parameter, data from two independent experiments were analysed by two-way ANOVA with "day" and "treatment" as the two factors. If the ANOVA demonstrated significant differences between treatments, Tukey's post hoc tests were run averaging over the two experiments, in order to determine which groups differed from the others. The cell numbers and cytokine levels were  $\log_{10}$ -transformed to achieve normally distributed data with equal variances. The following comparisons were

regarded: OVA + particles versus OVA alone and the respective particle alone, particle alone and OVA alone versus HBSS. Additionally, in the first set of experiments, the responses between all particles alone or between all OVA + particles were compared. In the second set of experiments, the coarse and fine fractions from the same sampling location were compared. Experimental groups were considered to differ if  $P \leq 0.05$ .

### 3. Results

#### 3.1. The primary cellular response to ambient air particles and diesel exhaust particles (DEP), with or without allergen

Five days after injection of allergen (OVA) and particles, separately or together, selected cellular parameters in the draining lymph node were measured. In this set of experiments, particle fractions which apparently induced different antibody responses with regard to the Th1 and Th2 balance were used: diesel exhaust particles (DEP), Rome fine (spring) and Oslo fine (summer, Oslo1) fractions.

##### 3.1.1. Cell numbers and cell phenotypes in the lymph node

In the flow cytometric analyses, three separate cell populations were observed in the light scatter dot plot (Fig. 1). These populations were mainly separated by different forward light scatter (FS), which to a large degree reflects the cell volume. The population with low FS comprised dead cells, as verified by propidium iodide staining, and was excluded from the analyses. The population with medium FS always contained more than 90% of the live cells, and the analyses of the surface molecules we present were performed gating on this main lymphocyte population. In agreement with Tuschl et al. (2002), the population with high FS was identified as strongly activated lymph node cells, with high baseline expression of surface molecules like MHC class II, CD86 and CD69 (data not shown). The relative number of cells in this population, hereafter called lymphoblasts, was calculated by dividing the number of lymphoblasts by the number of live lymphocytes.

As shown in Fig. 2A, the number of lymph node cells was significantly higher in the OVA + particle

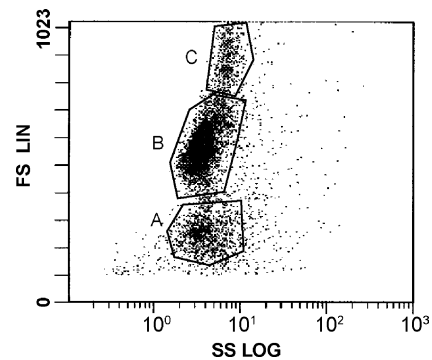


Fig. 1. Flow cytometric light scatter of an unstained lymph node cell suspension, 5 days after injection of 50 µg OVA with 100 µg Oslo1 particles. The x-axis represents the side scatter (SS) whereas the y-axis represents the forward scatter (FS). The three different gates used are indicated, gate A comprising the “dead cell population” excluded from the analyses, gate B comprising the main lymphocyte population, and gate C comprising the “lymphoblasts”.

groups than in the groups given OVA alone or the corresponding particle alone. Furthermore, Oslo1 and Rome particles alone, but not DEP, moderately but significantly increased the number of lymph node cells compared to HBSS. The relative number of CD19<sup>+</sup> cells (B lymphocytes) in the PLN was strongly increased by all particles with OVA compared to OVA alone or the corresponding particle alone (Fig. 2B). Correspondingly, the relative number of CD3<sup>+</sup> cells (T lymphocytes) was strongly decreased by OVA + particles (Fig. 2C). The different particles with OVA changed the relative numbers of B and T lymphocytes to different degrees, OVA + DEP significantly more than OVA + Oslo1 or OVA + Rome, and OVA + Oslo1 significantly more than OVA + Rome. Of the particles alone, only the Oslo1 particles significantly altered the relative numbers of B and T lymphocytes compared to HBSS. The relative number of T lymphocytes being CD4<sup>+</sup> was not affected by any of the particles, neither alone nor together with the allergen (data not shown). All three particle fractions with OVA increased the relative number of lymphoblasts compared to OVA alone and the corresponding particle alone (Fig. 2D). None of the particles alone altered the relative number of lymphoblasts to a statistically significant extent.

While all B lymphocytes expressed MHC class II molecules, the relative expression of MHC class II molecules (median fluorescence intensity) per B lymphocyte was markedly increased by OVA + particles

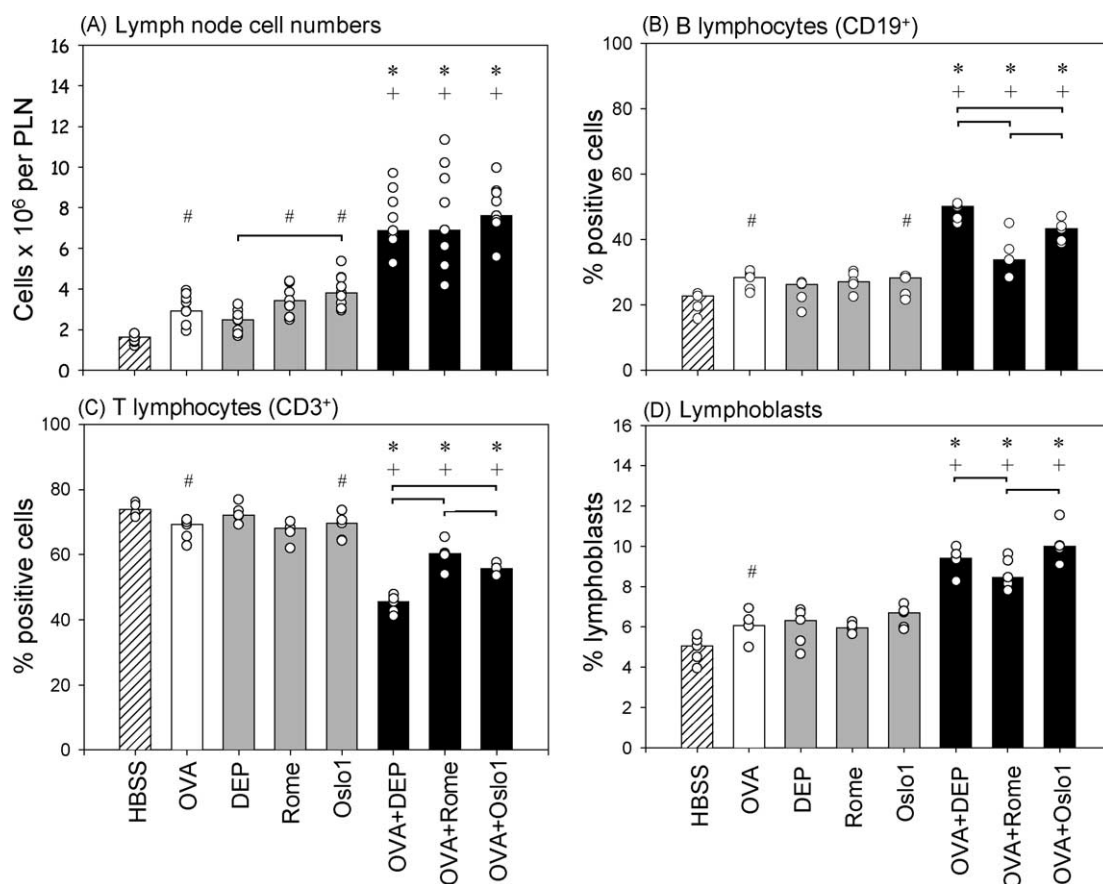


Fig. 2. Lymph node cell numbers and lymphocyte subpopulations in the popliteal lymph node 5 days after a single injection into both hind footpads of HBSS, 50  $\mu$ g OVA, 100  $\mu$ g DEP, Rome or Oslo1 particles alone or 50  $\mu$ g OVA with 100  $\mu$ g DEP, Rome or Oslo1 particles. Lymph node cell numbers (A) were measured on a Coulter cell counter. Single cell suspensions were stained with antibodies against CD19 (B) and CD3 (C), and the relative numbers of positive cells were determined by flow cytometry. The relative numbers of lymphoblasts (D) were determined by restrictive gating on the flow cytometric light scatter dot plot. Values for individual mice (circles) and median values (columns) for groups of nine (A) or five (B–D) mice are shown. Data from one representative experiment are shown, whereas the statistical analyses are based on two independent experiments. The cell number data were log-transformed before statistical analyses, to achieve normal distribution and equal variances. #, \* and + indicate a statistically significant difference ( $P < 0.05$ ) compared to HBSS, OVA and the corresponding particle, respectively. Brackets indicate a statistically significant difference ( $P < 0.05$ ) between the different particles.

compared to OVA alone or the corresponding particle alone (Fig. 3A). Furthermore, OVA + DEP increased the MHC class II expression significantly more than the two ambient air particles with OVA. Both Rome and Oslo1 particles alone marginally, but significantly, increased the MHC class II expression compared to DEP and HBSS. As shown in Fig. 3B, the relative number of CD86<sup>+</sup> B lymphocytes was significantly increased by OVA + DEP and OVA + Oslo1, but not by OVA + Rome, compared to OVA alone or particles

alone. Furthermore, OVA + DEP induced higher levels of CD86<sup>+</sup> B lymphocytes than the two ambient air particles with OVA, and OVA + Oslo1 induced higher levels than OVA + Rome. None of the particles alone affected the relative number of CD86<sup>+</sup> B lymphocytes. The relative number of CD23<sup>+</sup> B lymphocytes was generally high (more than 90% positive B lymphocytes in all groups), and further increased significantly after injection of OVA + particles (to about 97% positive B lymphocytes, data not shown). Because most B

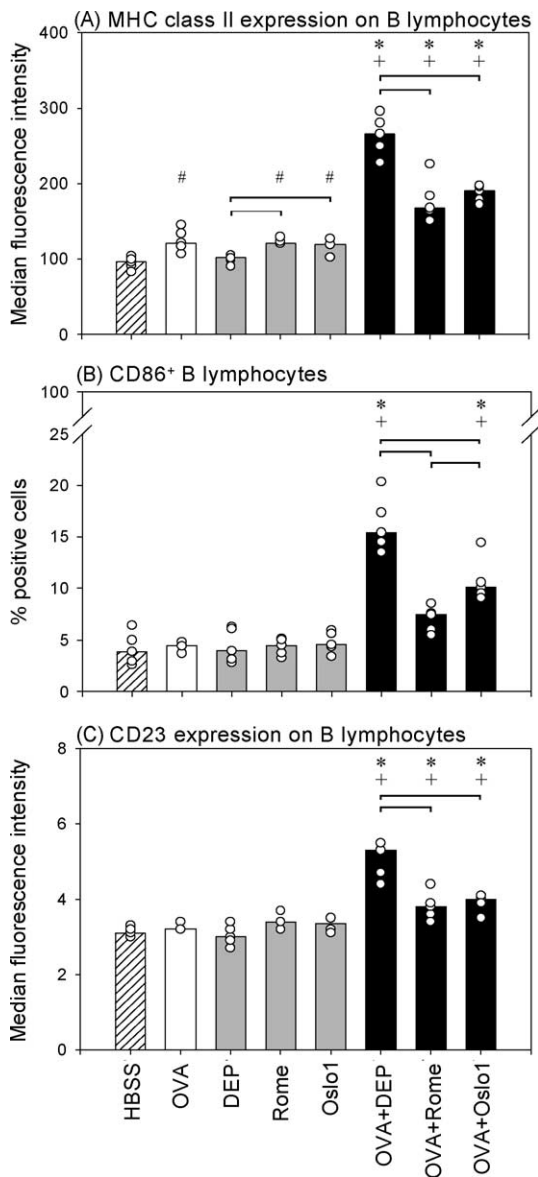


Fig. 3. Expression of surface molecules on B lymphocytes from the popliteal lymph node 5 days after a single injection into both hind footpads of HBSS, 50  $\mu$ g OVA, 100  $\mu$ g DEP, Rome or Oslo1 particles alone or 50  $\mu$ g OVA with 100  $\mu$ g DEP, Rome or Oslo1 particles. Single cell suspensions were double-stained with antibodies against CD19 and MHC class II (A), CD19 and CD86 (B) or CD19 and CD23 (C), and the median fluorescence intensity or the relative numbers of positive B lymphocytes (CD19<sup>+</sup>) were determined by flow cytometry. Values for individual mice (circles) and median values (columns) for groups of five mice are shown. Data from one representative experiment are shown, whereas the statistical analyses are based on two independent experiments. #, \* and + indicate a statistically significant

lymphocytes expressed CD23, alterations in the expression of CD23 per cell probably is of more biological relevance than the number of positive cells in this system. As shown in Fig. 3C, the expression of CD23 molecules per B lymphocyte was significantly increased by all particles with OVA compared to OVA alone and the corresponding particle alone. Again, OVA + DEP increased the expression significantly more than the two ambient air particles with OVA. None of the particles alone altered the CD23 expression compared to HBSS.

In general, OVA alone weakly affected the cell numbers and the measured cell surface markers, however not statistically significant for the expression of CD86 and CD23.

### 3.1.2. *Ex vivo* cytokine production by lymph node cells

After culture of the PLN cells with the mitogen ConA, the supernatant concentrations of IL-4 and IL-10 were markedly higher in the OVA + particle groups than in the groups given OVA alone or the corresponding particle alone (Figs. 4A and B). An exception was the levels in the OVA + Rome group, which were not significantly higher than OVA alone. Furthermore, OVA + DEP and OVA + Oslo1 induced significantly higher IL-10 levels than OVA + Rome. In the absence of OVA, Rome and Oslo1 particles significantly increased the levels of IL-4 or IL-10 compared to DEP and HBSS. Also OVA alone significantly increased the levels of IL-4 and IL-10. In contrast, the IFN- $\gamma$  levels were not significantly affected by any of the particles, neither alone nor together with OVA (Fig. 4C).

### 3.1.3. Lymph node histology

Five days after injection, the lymph nodes were markedly enlarged in the groups given OVA + particles compared to the control groups. Sections stained for macrophages, T and B lymphocytes illustrate how injection of particles together with allergen gave formation of germinal centers in the follicle areas of the lymph nodes (OVA and Oslo1 particles, alone or in combination, are shown as examples in Fig. 5A–C). Particles were observed scattered between

difference ( $P < 0.05$ ) compared to HBSS, OVA and the corresponding particle, respectively. Brackets indicate a statistically significant difference ( $P < 0.05$ ) between the different particles.

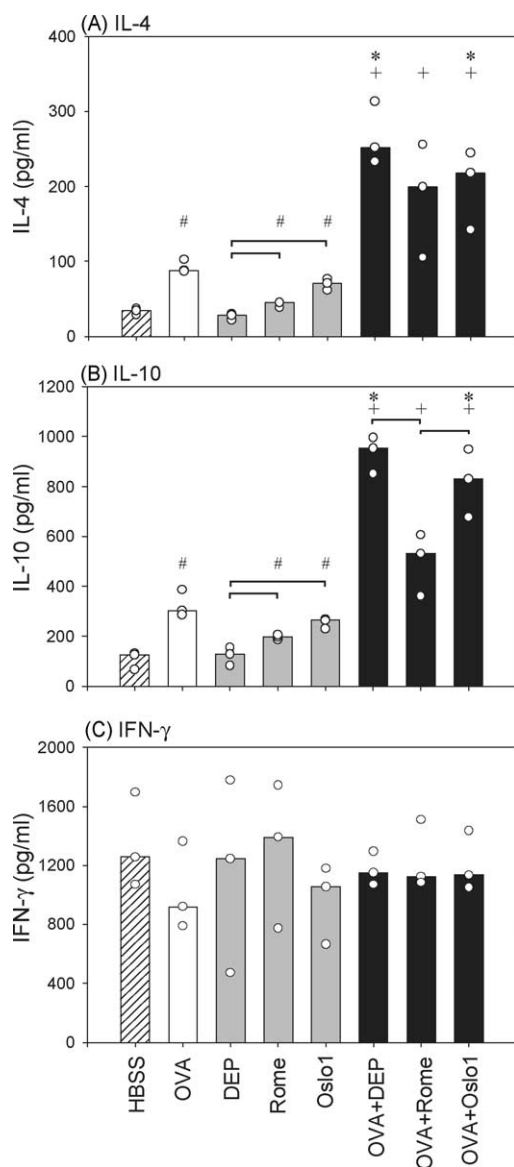


Fig. 4. Ex vivo cytokine production by lymph node cells on day 5 after a single injection into both hind footpads of HBSS, 50  $\mu$ g OVA, 100  $\mu$ g DEP, Rome or Oslo1 particles alone or 50  $\mu$ g OVA with 100  $\mu$ g DEP, Rome or Oslo1 particles. Single cell suspensions were cultured with ConA (5  $\mu$ g/ml) for 24 and 48 h. IL-4 (A), IL-10 (B) and IFN- $\gamma$  (C) in culture supernatants were measured by ELISA, and data from optimal time points are shown (24, 24 and 48 h, respectively). Each point represents a pool of cells from three animals and columns represent the group median value. Data from one representative experiment are shown, whereas the statistical analyses are based on two independent experiments. The cytokine levels were log-transformed before analyses to achieve normal distribution and

the lymphocytes in the lymph nodes, often localised within macrophage-like (acid phosphatase positive) cells (Fig. 5D). Five days after injection of fluorescent PSP with or without OVA, the PSP appeared to be associated with single cells, both within the T cell zone of the lymph nodes and in the marginal sinus of the lymph nodes (Fig. 5E).

### 3.2. Comparison of the primary cellular response to coarse and fine ambient air particle fractions with allergen

To examine the significance of particle fraction size, the same set of cellular parameters as described above was measured 5 days after injection of OVA with particles. The particles studied in this set of experiments were the coarse and fine fractions of the Amsterdam (spring), Lodz (summer) and Oslo (spring, Oslo2) fractions.

#### 3.2.1. Cell numbers and cell phenotypes in the lymph node

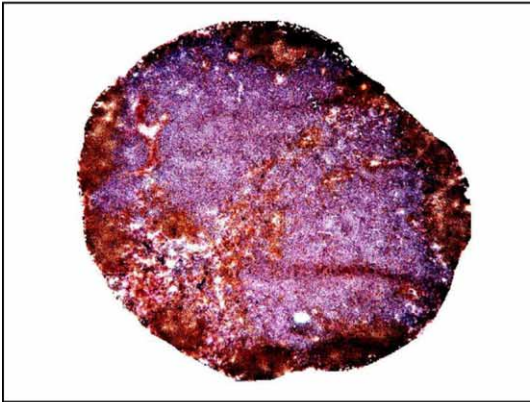
Overall, the number of PLN cells, the relative number of B lymphocytes and expression of MHC class II, CD86 and CD23 on B lymphocytes were significantly increased in all OVA + particle groups compared to the OVA group (selected parameters are shown in Fig. 6). No obvious systematic differences between the responses with the coarse and fine particle fractions were seen. However, the Lodz fine fraction with OVA increased the relative number of B cells and CD86<sup>+</sup> B lymphocytes significantly more than the Lodz coarse fraction with OVA (Fig. 6A and B).

#### 3.2.2. Ex vivo cytokine production by lymph node cells

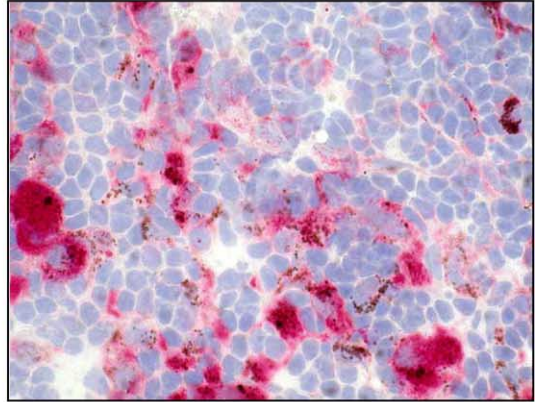
All coarse and fine particle fractions with OVA significantly increased the production of IL-4 (Fig. 6C) and IL-10 compared to OVA, without any systematic difference between the coarse and fine fractions. As shown in Fig. 6D, none of the particles with OVA significantly altered the IFN- $\gamma$  levels compared to OVA alone. However, the fine particle fractions with

equal variances. #, \* and + indicate a statistically significant difference ( $P < 0.05$ ) compared to HBSS, OVA and the corresponding particle, respectively. Brackets indicate a statistically significant difference ( $P < 0.05$ ) between the different particles.

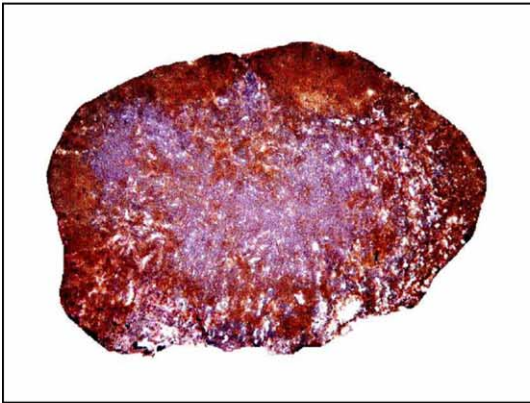
(A) OVA



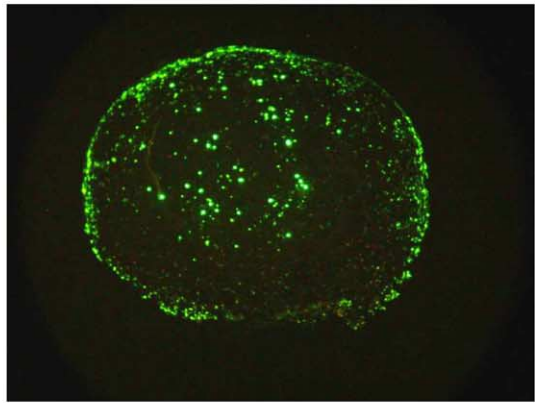
(D) OVA+Oslo1



(B) Oslo1



(E) OVA+PSP



(C) OVA+Oslo1

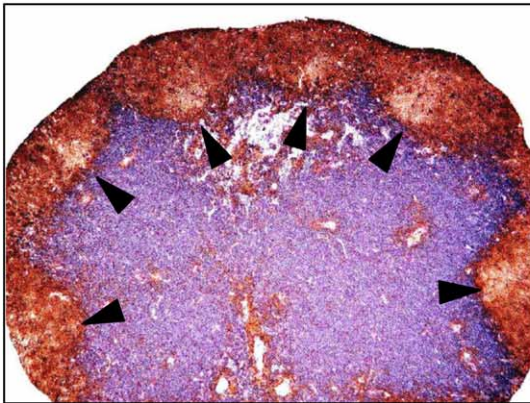


Fig. 5. Frozen sections of the draining lymph node 5 days after injection of 50 µg OVA (A), 100 µg Oslo1 particles (B), OVA + Oslo1 (C, D) and OVA + 0.1 µm fluorescent particles (PSP, 40 µg) (E). The sections shown in A–C were stained with antibodies against CD3 (T lymphocytes, purple colour), B220 (B lymphocytes, brown colour) and with naphthol ASBI phosphate and red-violet LB salt (macrophages, red colour). Germinal centers (GC) are indicated by arrows. The section shown in D was stained for macrophages (acid phosphatase active cells, red) and counterstained with hematoxylin. Ambient air particles of various sizes can be observed. Sample E was examined without any staining, using fluorescence microscopy.

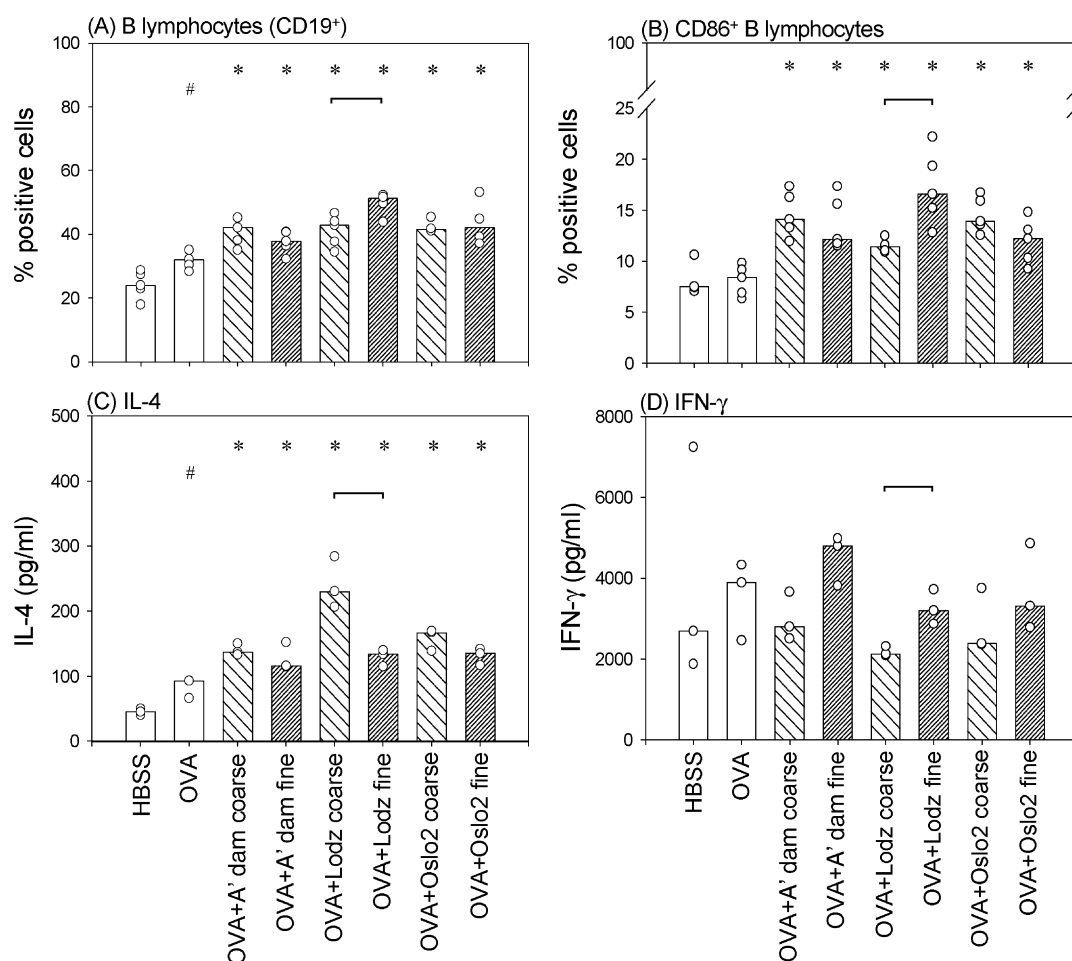


Fig. 6. Selected cellular parameters in the popliteal lymph node 5 days after a single injection into both hind footpads of HBSS, 50  $\mu$ g OVA or 50  $\mu$ g OVA with 100  $\mu$ g of the particle fractions Amsterdam (A'dam) coarse and fine, Lodz coarse and fine and Oslo2 coarse and fine. Single cell suspensions were stained with antibodies against CD19 (A), or CD19 and CD86 (B). IL-4 (C) and IFN- $\gamma$  (D) in cell culture supernatants were measured by ELISA after culture with ConA (5  $\mu$ g/ml) for 24 and 48 h, respectively. Each point represents an individual mouse (A, B) or a pool of cells from three animals (C, D), and columns represent the group median value. Data from one representative experiment are shown, whereas the statistical analyses are based on two independent experiments. The cytokine levels were log-transformed before statistical analyses, to achieve normal distribution and equal variances. # and \* indicate a statistically significant difference ( $P < 0.05$ ) compared to HBSS or OVA, respectively, whereas brackets indicate a statistically significant difference ( $P < 0.05$ ) between corresponding coarse and fine particle fractions with OVA.

OVA consistently tended to induce higher IFN- $\gamma$  levels than the coarse fractions, significantly only for the Lodz fractions.

#### 4. Discussion

In the present study, we have shown that ambient air particulate matter from four European cities enhanced

the primary cellular response to the allergen OVA in the draining lymph node after injection into the footpad. Co-injection of OVA and particles generally changed the expression of cell membrane molecules and altered the pattern of cytokine production in the lymph node cells. The cellular effects of particles and OVA given separately were marginal, indicating that the effect of particles on the primary cellular responses to OVA in the lymph node is synergistic, not just additive. This ad-

juvant effect of ambient air particles during the allergen sensitisation phase is associated with increased levels of allergen-specific antibody responses after allergen challenge, as demonstrated in other studies (Lovdal et al., 2003).

An adjuvant effect of different particles on IgE production has earlier been shown both after intraperitoneal, intranasal, intratracheal and subcutaneous exposure (Granum et al., 2001a, 2001b; van Zijverden et al., 2001; Lovik et al., 1997; Nygaard et al., 2004). Studies of the cellular response in the PLN after subcutaneous injection are therefore assumed to be relevant for the mechanisms of immunoregulation and the adjuvant effect of particles also after airways exposure. The subcutaneous PLN model is particularly well suited for studies of the cellular response in a draining lymph node, as the popliteal lymph node is the only node draining the footpad (Tilney, 1971). In addition, it is advantageous that the retained dose varies less after subcutaneous injection than after exposure by instillation or aerosol in the airways. Most RAIAP particle fractions have previously been found to have IgE and IgG2a adjuvant activity after subcutaneous injection into the footpad. Since no consistent differences were observed between cities or seasons (Lovdal et al., 2003), the RAIAP particle fractions examined in the present study were chosen based on their differing IgE and IgG2a adjuvant capacity.

Injection of particles together with OVA increased the PLN cell numbers and the relative number of B cells, and was associated with formation of germinal centers, which consist of highly proliferating B cells (Janeway et al., 1999). Correspondingly, we have recently shown that the absolute number of B cells was increased twice as much as the number of T cells 5 days after injection of PSP (0.1  $\mu\text{m}$  diameter) with OVA (Nygaard et al., 2004). In the lymph nodes, the ambient air particles and DEP seemed to be localised scattered between the lymphocytes, often localised within acid phosphatase positive cells. The fluorescent PSP appeared to co-localise with cells within the T cell zone and the marginal sinus of the lymph nodes. However, we cannot exclude that single PSP, which because of their small size would not be visible in the microscope, in addition were scattered extracellularly between the lymphocytes. Randolph et al. (1999) suggested that subcutaneously injected PSP were transported into the

T cell area of the draining lymph node by monocytes maturing into monocytic dendritic cells during migration. The observed particle-containing cells with acid phosphatase activity probably were antigen-presenting cells (APC). Most of the cellular parameters were weakly altered by OVA alone, and we suggest that the particles act by intensifying the inherent immune response towards OVA, most likely by acting on the APCs carrying the PSP and/or the allergen to the PLN. The adjuvant effect of particles could be due to the particles carrying a high amount of antigen into the APCs, and we have previously observed PSP-associated cells in the draining PLN from 1 day after injection (Nygaard et al., 2004). Further, there might be direct effects of the particles with their components on APC antigen processing and presentation (Hamilton et al., 2004). The particles may provide a “danger signal” (Matzinger, 2002) that stimulates the immune response. Also, there is the possibility that the particles induce a local inflammation at the injection site, thus increasing the number or the ability of the APC to take up antigen or migrate into the lymph node.

We have recently shown that the IgE adjuvant capacity of PSP of a wide size range (0.0588–11.14  $\mu\text{m}$  diameter) increases strongly with decreasing particle diameter. Furthermore, the PLN cellular response to OVA during the primary response gradually increased with decreasing PSP diameter, from no response with 11.14  $\mu\text{m}$  to a markedly increased response with 0.202  $\mu\text{m}$  PSP (Nygaard et al., in press). In contrast, although the fine RAIAP particle fractions overall induced higher OVA-specific antibody responses than the coarse fractions (Lovdal et al., 2003), no systematic differences between the lymph node effects of the coarse and fine particle fractions were observed. However, the different chemical components or physical particle characteristics may on the individual sample level obscure trends that would become more clear with a larger number of samples or different parameters. For the present samples, the cellular parameters investigated could not predict the quantitative differences observed in antibody responses for the different ambient particle size fractions. However, in relation to the role IFN- $\gamma$  may play in worsening and persistence of asthmatic inflammation (Rowe et al., 2004), it is of interest that the fine particle fractions with OVA consistently induced higher IFN- $\gamma$  levels than the coarse particle fractions. Epidemiological findings indicate that

fine particles are more associated with acute asthma symptoms than coarse particles (Schwartz and Neas, 2000).

DEP have been shown to exclusively exert a Th2-associated antibody adjuvant effect after subcutaneous injection in BALB/cA mice (Lovik et al., 1997; van Zijverden et al., 2000; Nygaard et al., in press), whereas the RAIAP particles have been shown to increase both the Th2- and Th1-associated antibody levels (Lovdal et al., 2003). The antibody response pattern with the fine particles DEP, Oslo1 and Rome was Th2, Th1 > Th2 and Th2 > Th1, respectively. In the present study, the cellular responses to OVA were most strongly enhanced by DEP, and more by Oslo1 particles than by Rome particles. The particle-induced alterations of the cellular parameters studied here, including the cytokines, did not predict the individual particle sample's potency to skew the Th1 or Th2 antibody balance observed in the antibody experiments (Lovdal et al., 2003). The polarised Th2 adjuvant DEP induced markedly more pronounced changes in most of the cellular parameters than did the mixed Th1 and Th2 adjuvants Rome and Oslo1 particles. Assessing the effect of particles on regulatory T cells could possibly help explain the antibody response pattern.

Even though DEP with OVA induced clearly more pronounced cellular changes than the RAIAP particles, DEP alone appeared not to affect the cellular parameters measured. Similarly, we have previously shown that 0.1 µm PSP, another Th2-polarised antibody adjuvant, when given alone did not detectably alter the primary response in the lymph node (Nygaard et al., 2004). In contrast, the Oslo1 and Rome particles alone increased the lymph node cell numbers and MHC class II expression, as well as IL-4 and IL-10 levels. This effect of the ambient air particles is in accordance with the findings of Becker and Soukup (2003). These authors found that coarse, fine and ultrafine ambient air particles, but not DEP or volcanic ash, in vitro increased the expression of HLA-DR, CD40 and CD86 on human blood-derived monocytes. Effects of particles on cytokine production have been shown in humans, where DEP increased a broad array of nasal cytokine expression, including IL-4 and IL-10 (Diaz-Sanchez et al., 1996). Since PAHs, including extracts from DEP, pyrene and phenanthrene, in vitro have been found to up-regulate IL-4 transcription and protein production (Bommel et al., 2000; Devouassoux et al., 2002), the

particles' varying capacity to increase the cytokine levels probably is influenced by differing substances adsorbed to the particles. The ability of the particles to induce production of different cytokines will presumably influence the skewing of the responses towards Th1- or Th2-associated antibodies.

Taken together, we have shown that ambient air particles enhance the cellular response in the draining lymph node during the primary response to an allergen. The particles caused up-regulation of several parameters in part associated with an immune response in general (PLN cell numbers and B cell expression of MHC class II), in part associated with a Th2-related immune response (the cytokine IL-4), as well as parameters possibly involved in the regulation of the Th2 response (CD86 and CD23 expression). The cellular parameters measured could, for the individual sample, neither predict the degree of a Th2- or Th1-skewed antibody response, nor the stronger antibody adjuvant effect of fine than coarse particle fractions from ambient air.

## Acknowledgements

We thank Else-Carin Groeng, Bodil Hasseltvedt, Åse Eikeset and Berit A. Stensby for invaluable technical assistance. We also appreciate the help of Ellen Namork and Astri Grestad with the electron microscopy analyses. This work was supported by the Norwegian Research Council, grant no. 129593/310 and the RAIAP project (Respiratory Allergy and Inflammation due to Ambient Particles)—A European Commission Shared-Cost Research Project, QLK-CT-2000-00792.

## References

- Atkinson, R.W., Anderson, H.R., Sunyer, J., Ayres, J., Baccini, M., Vonk, J.M., Boumghar, A., Forastiere, F., Forsberg, B., Touloumi, G., Schwartz, J., Katsouyanni, K., 2001. Acute effects of particulate air pollution on respiratory admissions: results from APHEA 2 project. *Air Pollution and Health: a European Approach*. Am. J. Respir. Crit. Care Med. 164, 1860–1866.
- Becker, S., Soukup, J., 2003. Coarse (PM<sub>2.5-10</sub>), fine (PM<sub>2.5</sub>), and ultrafine air pollution particles induce/increase immune costimulatory receptors on human blood-derived monocytes but not on alveolar macrophages. *J. Toxicol. Environ. Health A* 66, 847–859.

- Bommel, H., Li-Weber, M., Serfling, E., Duschl, A., 2000. The environmental pollutant pyrene induces the production of IL-4. *J. Allergy Clin. Immunol.* 105, 796–802.
- Cassee, F.R., Fokkens, P.H.B., Leseman, D.L.A.C., Bloemen, H.J.T., Boere, A.J.F., 2003. Respiratory allergy and inflammation due to ambient air particles (RAIAP). Collection and characterisation of particulate matter samples from 5 European sites. Report 863001001/2003. RIVM, Bilthoven.
- Devouassoux, G., Saxon, A., Metcalfe, D.D., Prussin, C., Colomb, M.G., Brambilla, C., Diaz-Sanchez, D., 2002. Chemical constituents of diesel exhaust particles induce IL-4 production and histamine release by human basophils. *J. Allergy Clin. Immunol.* 109, 847–853.
- Diaz-Sanchez, D., Tsien, A., Casillas, A., Dotson, A.R., Saxon, A., 1996. Enhanced nasal cytokine production in human beings after in vivo challenge with diesel exhaust particles. *J. Allergy Clin. Immunol.* 98, 114–123.
- Dybing, E., Lovdal, T., Hetland, R.B., Lovik, M., Schwarze, P.E., 2004. Respiratory allergy adjuvant and inflammatory effects of urban ambient particles. *Toxicology* 198, 307–314.
- Ebelt, S., Brauer, M., Cyrys, J., Tuch, T., Kreyling, W.G., Wichmann, H.E., Heinrich, J., 2001. Air quality in postunification Erfurt, East Germany: associating changes in pollutant concentrations with changes in emissions. *Environ. Health Perspect.* 109, 325–333.
- Granum, B., Gaarder, P.I., Groeng, E., Leikvold, R., Namork, E., Lovik, M., 2001a. Fine particles of widely different composition have an adjuvant effect on the production of allergen-specific antibodies. *Toxicol. Lett.* 118, 171–181.
- Granum, B., Gaarder, P.I., Lovik, M., 2001b. IgE adjuvant effect caused by particles—immediate and delayed effects. *Toxicology* 156, 149–159.
- Granum, B., Lovik, M., 2002. The effect of particles on allergic immune responses. *Toxicol. Sci.* 65, 7–17.
- Gutting, B.W., Updyke, L.W., Amacher, D.E., 2002. Diclofenac activates T cells in the direct popliteal lymph node assay and selectively induces IgG1 and IgE against co-injected TNP-OVA. *Toxicol. Lett.* 131, 167–180.
- Hamilton Jr., R.F., Holian, A., Morandi, M.T., 2004. A comparison of asbestos and urban particulate matter in the in vitro modification of human alveolar macrophage antigen-presenting cell function. *Exp. Lung Res.* 30, 147–162.
- ISAAC (The International Study of Asthma and Allergies in Childhood Steering Committee), 1998. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. *Lancet* 351, 1225–1232.
- Janeway, C.A., Travers, P., Walport, M., Capra, J.D., 1999. The humoral immune response. In: *Immunobiology*. Elsevier Science, London, pp. 307–361.
- Jogi, R., Janson, C., Bjornsson, E., Boman, G., Bjorksten, B., 1998. Atopy and allergic disorders among adults in Tartu, Estonia compared with Uppsala Sweden. *Clin. Exp. Allergy* 28, 1072–1080.
- Katsouyanni, K., Touloumi, G., Samoli, E., Gryparis, A., Le Tertre, A., Monopolis, Y., Rossi, G., Zmirou, D., Ballester, F., Boumghar, A., Anderson, H.R., Wojtyniak, B., Paldy, A., Braunstein, R., Pekkanen, J., Schindler, C., Schwartz, J., 2001. Confounding and effect modification in the short-term effects of ambient particles on total mortality: results from 29 European cities within the APHEA2 project. *Epidemiology* 12, 521–531.
- Lovdal, T., Groeng, E., Dybing, E., Lovik, M., 2003. Respiratory allergy and inflammation due to ambient particles (RAIAP), a European-wide assessment. *Allergy screening. Toxicol. Sci.* 72 (Suppl.), 593, Abstract.
- Lovik, M., Hogseth, A.K., Gaarder, P.I., Hagemann, R., Eide, I., 1997. Diesel exhaust particles and carbon black have adjuvant activity on the local lymph node response and systemic IgE production to ovalbumin. *Toxicology* 121, 165–178.
- Maejima, K., Tamura, K., Taniguchi, Y., Nagase, S., Tanaka, H., 1997. Comparison of the effects of various fine particles on IgE antibody production in mice inhaling Japanese cedar pollen allergens. *J. Toxicol. Environ. Health* 52, 231–248.
- Matzinger, P., 2002. The danger model: a renewed sense of self. *Science* 296, 301–305.
- Nygaard, U.C., Samuelsen, M., Aase, A., Lovik, M., in press. The capacity of particles to increase allergic sensitization is predicted by particle number and surface area, not by particle mass. *Toxicol. Sci.*
- Nygaard, U.C., Ormstad, H., Aase, A., Lovik, M., 2004. The IgE adjuvant effect of particles: characterisation of the primary cellular response in the draining lymph node. *Toxicology* 206, 181–193.
- Parnia, S., Brown, J.L., Frew, A.J., 2002. The role of pollutants in allergic sensitization and the development of asthma. *Allergy* 57, 1111–1117.
- Randolph, G.J., Inaba, K., Robbiani, D.F., Steinman, R.M., Muller, W.A., 1999. Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. *Immunity* 11, 753–761.
- Rowe, J., Heaton, T., Kusel, M., Suriyaarachchi, D., Serralha, M., Holt, B.J., de Klerk, N., Sly, P.D., Holt, P.G., 2004. High IFN- $\gamma$  production by CD8<sup>+</sup> T cells and early sensitization among infants at high risk of atopy. *J. Allergy Clin. Immunol.* 113, 710–716.
- Schafer, T., Ring, J., 1997. Epidemiology of allergic diseases. *Allergy* 52 (Suppl. 38), 14–22.
- Schwartz, J., Neas, L.M., 2000. Fine particles are more strongly associated than coarse particles with acute respiratory health effects in schoolchildren. *Epidemiology* 11, 6–10.
- Sedgwick, J.D., Holt, P.G., 1985. Induction of IgE-secreting cells and IgE isotype-specific suppressor T cells in the respiratory lymph nodes of rats in response to antigen inhalation. *Cell. Immunol.* 94, 182–194.
- Tilney, N.L., 1971. Patterns of lymphatic draining in the adult laboratory rat. *J. Anat.* 109, 369–383.
- Tuschl, H., Landsteiner, H.T., Kovac, R., 2002. Application of the popliteal lymph node assay in immunotoxicity testing: complementation of the direct popliteal lymph node assay with flow cytometric analyses. *Toxicology* 172, 35–48.
- van Zijverden, M., van der Pijl, A., Bol, M., van Pinxteren, F.A., de Haar, C., Penninks, A.H., van Loveren, H., Pieters, R., 2000. Diesel exhaust, carbon black, and silica particles display distinct Th1/Th2 modulating activity. *Toxicol. Appl. Pharmacol.* 168, 131–139.
- van Zijverden, M., de Haar, C., van Beelen, A., van Loveren, H., Penninks, A.H., Pieters, R., 2001. Coadministration of antigen and particles optimally stimulates the immune response in an in-

- tranasal administration model in mice. *Toxicol. Appl. Pharmacol.* 177, 174–178.
- von Mutius, E., Fritzsche, C., Weiland, S.K., Roll, G., Magnussen, H., 1992. Prevalence of asthma and allergic disorders among children in united Germany: a descriptive comparison. *BMJ* 305, 1395–1399.
- von Mutius, E., Martinez, F.D., Fritzsche, C., Nicolai, T., Roell, G., Thiemann, H.H., 1994. Prevalence of asthma and atopy in two areas of West and East Germany. *Am. J. Respir. Crit. Care Med.* 149, 358–364.
- Williams, M.L., 1999. Patterns of air pollution in developed countries. In: Holgate, S.T., et al. (Eds.), *Air Pollution and Health*. Academic Press, London, pp. 83–104.
- Zinkernagel, R.M., 2000. Localization dose and time of antigens determine immune reactivity. *Semin. Immunol.* 12, 163–171.