Toxicology 261 (2009) 103–111

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Contact and respiratory sensitizers can be identified by cytokine profiles following inhalation exposure

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ARTICLE INFO

Article history:
Received 17 February 2009
Received in revised form 7 April 2009
Accepted 28 April 2009
Available online 5 May 2009

Keywords:
Sensitizers
Inhalation exposure
Skin exposure
Lymph node responses
Cytokine profiles

ABSTRACT

There are currently no validated animal models that can identify low molecular weight (LMW) respiratory sensitizers. The Local Lymph Node Assay (LLNA) is a validated animal model developed to detect contact sensitizers using skin exposure, but all LMW respiratory sensitizers tested so far were also positive in this assay. Discrimination between contact and respiratory sensitizers can be achieved by the assessment of cytokine profiles. In a LLNA using the inhalation route, both contact and respiratory sensitizers enhanced proliferation in the draining lymph nodes. The question was if their cytokine profiles were affected by the route of exposure. Male BALB/c mice were exposed head/nose-only during 3 consecutive days to the respiratory sensitizers trimellitic anhydride, phthalic anhydride, toluene diisocyanate, hexamethylene diisocyanate (HDI), and isophorone diisocyanate; the contact sensitizers dinitrochlorobenzene (DNCB), oxazolone (OXA) and formaldehyde (FA), and the irritant methyl salicylate (MS). Three days after the last exposure the draining lymph nodes were excised and cytokine production was measured after *ex vivo* stimulation with Concanavalin A. Skin application was used as a positive control. After inhalation exposure the respiratory sensitizers induced more interleukin-4 (IL-4) and interleukin (IL-10) compared to the contact sensitizers, whereas the contact sensitizers, except formaldehyde, induced relatively more interferon-γ (IFN-γ) production. When IL-4 and IFN-γ were plotted as a function of the proliferative response, it was shown that IL-4 could be used to identify respiratory sensitizers, except HDI, at concentration levels inducing intermediate stimulation indices. HDI could be distinguished from DNCB and OXA at high SI values. In contrast, contact sensitizers could only be identified when IFN-γ was measured at high stimulation indices. The skin positive control, tested at high concentrations, showed comparable results for IL-4 and IL-10, whereas IFN-γ levels could not be used to discriminate between respiratory and contact sensitizers. The contact sensitizer FA and the irritant MS did not induce significant cytokine production after inhalation and skin exposure. In conclusion, the respiratory LLNA is able to identify and distinguish strong contact and respiratory sensitizers when simultaneously proliferation and cytokine production are assessed in the upper respiratory tract draining LNs.

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1. Introduction

At present, there are no widely accepted and validated test methods to identify low molecular weight (LMW) respiratory sensitizers, i.e. compounds that are considered capable of inducing allergic airway reactions including asthma and rhinitis. Most exploratory models investigated so far have been using strong respiratory sensitizers. Moreover, these models detected the potential of a chemical to induce respiratory sensitization at relatively high concentrations. Consequently, the sensitivity of the used models is unknown, and these do not provide information on low exposure concentrations generally encountered, and on threshold levels to be used in risk assessment. In addition, the various models used different application routes, i.e. intradermal, topical, intranasal or inhalation exposure, and assessed different parameters (reviewed by Arts and Kuper, 2007). Therefore, standardized and validated test models and methods are urgently needed in order to identify LMW respiratory sensitizers and to recommend non-hazardous exposure levels for consumers and workers.

Currently under REACH, the Local Lymph Node Assay (LLNA; OECD, 2002) is the method of choice for the evaluation of the skin sensitizing potential of LMW chemicals (EC, 2006). The LLNA does

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doi:10.1016/j.tox.2009.04.057

not evaluate an antigen specific response based on sensitization and subsequent challenge, as the formerly recommended guinea pig maximization test (GPMT) or Buehler occluded patch assay. In contrast, the LLNA is based on an increase of cellular proliferation indicating the induction of an immune response. When a chemical induces a stimulation index (SI) of 3 or higher in the LLNA it is considered a sensitizer (Basketter et al., 1999a,b; Haneke et al., 2001; Kimber and Basketter, 1992). To date, most if not all of the respiratory sensitizers tested so far showed a positive response in the LLNA or in other assays for skin sensitization such as the guinea pig maximization or the Buehler test, identifying these compounds as sensitizers (Arts, 2001; Kimber et al., 2007; Van Loveren et al., 2008).

It has been shown that discrimination between contact and respiratory sensitizers is possible by assessing cytokine profiles in the draining lymph nodes using different types of protocols (Dearman et al., 1995; Plitnick et al., 2002; Vandebriel et al., 2000, 2003; Van Och et al., 2002). After dermal exposure, contact sensitizers induced mainly IFN-γ and low or no IL-4 production. Respiratory sensitizers induced a considerable amount of not only IL-4 but also IFN-γ. Therefore, the induction of the Th2 cytokine IL-4 was suggested to be indicative for respiratory sensitizers. Other cytokines used for the indication of Th2 responses were IL-5, IL-10 and IL-13 (Aronica et al., 1999; Dearman et al., 1996; Farraj et al., 2006; Plitnick et al., 2002). In addition to IFN-γ induction, also IL-12 induction was reported for identifying Th1 responses in mice (Dearman et al., 1999, 2002).

Although strong LMW respiratory sensitizers have been identified and distinguished from contact sensitizers by measuring cytokine production, the skin as route of exposure might not be the most appropriate one to detect respiratory sensitizers. The immune response induced by LMW chemicals can be different in the skin and lung, for instance due to differences in antigen presentation. Therefore, a LLNA was performed using the inhalation route, with the aim to explore if the route of exposure was of influence on proliferation and cytokine responses after inhalation exposure to both contact and respiratory sensitizers. The method was based on the protocol of the modified LLNA, in which proliferation is measured *ex vivo*, by adding the radioactive label to the cultured lymph node (LN) cells (De Jong et al., 2002; Kimber and Weisenberger, 1989; Piccotti et al., 2006; Piccotti and Kawabata, 2008; Van Och et al., 2000). A detailed description of the design of the respiratory LLNA, the pathology of the respiratory tract, and the proliferation responses in lymph nodes draining the respiratory tract following inhalation exposure to LMW chemicals is reported elsewhere (Arts et al., 2008). In these series of experiments, the mandibular and auricular lymph node cells were isolated for determination of both the cellular proliferation and cytokine production. It was shown that both respiratory and contact sensitizers provoked a proliferative response in the mandibular lymph nodes but the potency ranking differed from that in the skin LLNA (Arts et al., 2008). Here we report the effects of the inhalation exposure to respiratory and contact sensitizers on cytokine profiles.

2. Materials and methods

2.1. Animals

Young adult (6–7 weeks of age) male inbred BALB/c mice were obtained from Charles River Deutschland (Sulzfeld, Germany) and acclimatized for at least 5 days before the start of the study. BALB/c mice show similar responses in the LLNA compared to CBA/Ca mice (Woolhiser et al., 2000). The animals were bred under specified pathogen-free (SPF) conditions. During the experiments, the animals were housed barrier maintained under conventional conditions in light-, humidity-, and temperature-controlled rooms. All animals were housed 3 or 6 per group in macrolon cages. The mice were fed a standard pellet diet (RM3 [E] SQC, Special Diet Service, Witham, UK) and unfluoridated tap water *ad libitum*. All other husbandry conditions were maintained according to all applicable provisions of the following national laws: Experiments on Animals Decree, and Experiments on Animals Act. All animal experiments were performed according to all applicable national laws, and had permission from the TNO Commission of Animal Welfare.

2.2. Chemicals and exposure

The chemicals investigated belong to the group of well-known contact and respiratory sensitizers. These included trimellitic anhydride (TMA; 97% purity; Aldrich, Brussels, Belgium), toluene-diisocyanate (TDI; technical grade, mixture of 80% 2,4-TDI and 20% 2,6-TDI; Aldrich), hexamethylene-1,6-diisocyanate (HDI; 98% purity; Aldrich), 2,4-dinitrochlorobenzene (DNCB; purity at least 98%; Sigma, St. Louis, MO, USA), oxazolone (OXA; 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; purity 99.6%; Sigma), phthalic anhydride (PA; purity at least 99%; Fluka, Buchs, Switzerland), isophorone diisocyanate (IPDI, Degussa GmbH, Marl, Germany), and formaldehyde (FA, 36.5% in water; Fluka). Methyl salicylate (MS; purity at least 99%; Sigma), a respiratory and skin irritant, was used as negative control. Other chemicals used were acetone (Biosolve, Valkenswaard, The Netherlands) and raffinated olive oil (Sigma).

2.3. Experimental design respiratory LLNA

A series of experiments was performed using head/nose-only inhalation exposure of mice to the various LMW compounds. The analysis of cytokine induction was performed in the same mice of which the results on cell proliferation and respiratory tract pathology were reported elsewhere (Arts et al., 2008). Mice were randomly allocated to the experimental groups; 6 animals for each experimental group and 6 or 12 animals for the vehicle control groups. Mice were restrained in Battelle tubes and exposed to the test or control atmospheres in a head/nose-only inhalation unit. Mice were exposed to a fixed concentration of the chemical and the exposure duration was varied in order to obtain an increasing exposure dose. Mice were exposed for 45, 90, 180 or 360 min/day on 3 consecutive days (days 0, 1, and 2). The following respiratory sensitizers were used: TMA (30 mg/m^3), PA (15 mg/m^3), TDI (7.5 mg/m^3), HDI (7.5 mg/m^3), and IPDI (7.5 mg/m^3). Contact sensitizers included DNCB (30 mg/m^3), OXA (15 mg/m^3) and FA (3.6 mg/m^3). In addition, the respiratory and skin irritant MS (30 mg/m^3) was used. Besides the IPDI mentioned above, which was in monomeric form, also an oligomeric form of IPDI (15 mg/m^3) was tested (Arts et al., 2008). As this compound did not induce proliferation in the draining lymph nodes, did not induce histopathological changes in the respiratory tract, nor any increase in cytokine levels; no data on this chemical are reported.

For inhalation exposure, the chemicals were evaporated in air without solvent (TDI, HDI, FA and MS) or first dissolved in acetone and nebulized in air, i.e. test atmospheres were generated as aerosols (TMA, PA, IPDI, DNCB, OXA). Acetone was used to help generate test atmospheres of respirable particles of compounds normally consisting of very large non-inhalable particles. Controls were exposed to air only (in case of evaporation of the test substance without solvent) or air containing at most 0.06% acetone (when the test chemical was dissolved in acetone) for 360 min/day. An extended description of the generation of the test atmospheres and analyses of the actual concentrations inhaled by the animals is reported elsewhere (Arts et al., 2008).

2.4. Experimental design skin LLNA

The dermal route (ear application; $n = 3$) was used as a positive control, using a single high concentration. In short, 25 μl of the chemical dissolved in acetone:olive oil (4:1) solution (AOO) was applied on the dorsum of both ears (50 μl per animal) for 3 consecutive days (days 0, 1, and 2). Controls ($n = 6$) were exposed to the vehicle, AOO, only. Concentrations for skin exposure (w/v) were: TMA (50%), PA (25%), TDI (1%), HDI (1%), IPDI (1%), DNCB (1%), OXA (0.1%), and MS (25%). FA was used as 10% (v/v). On day 5, auricular LNs were collected and used for *ex vivo* cell proliferation and cytokine measurements.

2.5. Collection of lymph nodes after inhalation or dermal exposure

Three days after the last inhalation exposure, LNs were collected and used for *ex vivo* cell proliferation (see Arts et al., 2008) and cytokine measurements. In preliminary studies with a high concentration of TMA (250 mg/m^3), the most responsive LNs were established to be the mandibular LNs. Therefore, these LNs were taken out in all further experiments; only when grossly observed enlargement of other LNs was noted with a given test compound, these lymph nodes were collected additionally. This was the case for the posterior cervical LNs in mice exposed by inhalation to HDI and IPDI and for the auricular LNs following inhalation exposure to TDI, HDI, IPDI and OXA. For a better comparison, the auricular LNs were taken out following all exposures, except for DNCB, which was tested already before the tests with diisocyanates and OXA.

2.6. Preparation of single cell suspensions

The left and right auricular, or left and right mandibular LNs were taken out and pooled for each animal. Single cell suspensions were prepared in supplemented medium (RPMI 1640 (Gibco, Life Technologies, Breda, The Netherlands)

with 100 U/ml penicillin and 100 μg/ml streptomycin) to which 5% heat inactivated Fetal Calf Serum (FCS) (Integro, Zaandam, The Netherlands) was added. LNs were pressed trough a 70 μm nylon cell strainer (Falcon, Franklin Lakes, USA). The cells were washed in supplemented medium with 5% FCS (10 min, 300 *g*, 4 °C) and resuspended in 1 ml supplemented medium with 10% FCS. After counting the cells in a Coulter Counter (Z2, Coulter Electronics, Mijdrecht, The Netherlands) the concentration of the cell suspensions was adjusted to 1×10^7 cells/ml. When necessary, cell suspensions of several animals were pooled in order to obtain cell concentrations of 1×10^7 cells/ml, notably so for vehicle (air or AOO) treated controls.

Cell suspensions were used for determination of proliferation (a detailed description of the methods can be found in Arts et al., 2008) as well as for cytokine production after culturing with Con A. Stimulation indices (SIs) were calculated by dividing the ^{3}H-methylthymidine ([^{3}H]-TdR) incorporation of the experimental group with the mean [^{3}H]-TdR incorporation of the vehicle group. The SI values after respiratory exposure were calculated by comparing the mean results to that of the air-exposed control group; the SI value after dermal exposure was calculated by using the dermal vehicle control group.

2.7. Assessment of cytokine production

Production of cytokines by activated LN cells was determined after culturing the isolated LN cells (1×10^6 cells/ml) with Concanavalin A (Con A, 5 μg/ml) for 24 h. After 24 h, the supernatants were harvested and levels of interferon-γ (IFN-γ), interleukin 4 (IL-4), interleukin 10 (IL-10) and interleukin 12 (IL-12 (p70)) were determined with a Bio-Plex assay (BioRad Laboratories, Hercules, California). With this assay, multiple cytokines can be measured in each sample at the same time. The method is a bead-based multiplex assay, in which the antibodies are bound to fluorescent beads. The beads differ in fluorescence spectrum, making it possible to measure various cytokines at the same time. Fluorescence was measured on a Luminex® (Biorad Life Science) and Luminex software was used to calculate the amount of cytokines (in pg/ml supernatant) using standard reference curves, included in the assay. Cytokine production was expressed in pg per 1×10^6 cells, i.e. the response (in pg/ml) was divided by the cell culture concentration (1×10^6 cells/ml). Cytokine levels below 5 pg per 10^6 cells were considered to be negative.

2.8. Data analysis and statistics

Data are presented as means with their standard errors. Statistical analysis was performed with SPSS software (SPSS Inc., Chicago, IL, USA). SIs in mandibular and auricular LNs following inhalation exposure were statistically analyzed after logarithmic transformation, using a one-way ANOVA, followed by a two-sided Dunnett's test. The differences in cytokine levels between the vehicle group and groups exposed either by inhalation or by topical application with the different chemicals were statistically tested with a two-sided Student's *t*-test. A one-sided Student's *t*-test was used to determine statistical differences in IL-4 and IFN-γ production induced by contact and respiratory sensitizers at intermediate and high stimulation indices.

The significance level was set at $p < 0.05$. Before statistical analysis, outliers were detected using Grubb's test and rejected for further analysis.

3. Results

3.1. Cell proliferation

The results of the draining (mandibular) lymph node responses are summarized in Table 1. An extensive evaluation of these lymph node responses and pathology of the respiratory tract are reported elsewhere (Arts et al., 2008). In summary, all respiratory sensitizers investigated, TMA, PA, TDI, HDI and IPDI, as well as the contact sensitizers DNCB and OXA induced a positive proliferation response in the mandibular LNs (SI > 3). The contact sensitizer FA and the irritant MS did not induce a positive response. Inhalation exposure to TDI, HDI, IPDI and OXA stimulated auricular LNs as well, inducing even higher SI values than in the mandibular LNs.

The positive skin exposure control identified all sensitizers (SI > 3) and was negative for the irritant MS.

3.2. IL-4 induction

IL-4 production after inhalation exposure is presented in Fig. 1a. Cytokine production expressed as pg per lymph node was also calculated, but since the cytokine profiles were similar, only graphs showing cytokines in pg per 10^6 cells are shown. All respiratory sensitizers investigated, TMA, PA, TDI, HDI and IPDI, and the contact sensitizers DNCB and OXA induced a positive IL-4 response in de mandibular LN, while FA and MS did not (Fig. 1a). TMA and PA induced a bell-shaped curve; the highest levels of IL-4 were produced after exposure for 90 min per day on 3 consec-

Table 1
Effect of inhalation and skin exposure on cellular proliferation expressed as stimulation indices (SIs) in draining lymph nodes.

	Inhalation exposure					Skin exposure
	Vehicle control	45 min/day	90 min/day	180 min/day	360 min/day	
Mandibular lymph nodes						
TMA (30, 50)[a]	1.0 ± 0.14 (11)[b]	2.0 ± 0.26 (6)*	5.6 ± 1.26 (6)**	2.3 ± 0.37 (5)**	2.0 ± 0.20 (5)*	nd[c]
PA (15, 25)	1.0 ± 0.10 (11)	2.9 ± 0.57 (6)***	3.6 ± 0.54 (5)***	2.3 ± 0.15 (5)**	2.9 ± 0.52 (6)***	nd
TDI (7.5, 1)	1.0 ± 0.12 (11)	3.3 ± 0.67 (6)***	3.4 ± 0.98 (6)***	5.5 ± 1.35 (6)***	3.2 ± 0.23 (6)***	nd
HDI (7.5, 1)	1.0 ± 0.05 (5)	4.7 ± 1.04 (6)***	6.0 ± 0.95 (6)***	6.5 ± 1.13 (6)***	9.8 ± 1.02 (6)***	nd
IPDI (7.5, 1)	1.0 ± 0.19 (5)	2.8 ± 0.84 (6)	3.4 ± 0.99 (5)*	4.4 ± 0.53 (5)**	4.0 ± 0.76 (5)*	nd
DNCB (30, nd)	1.0 ± 0.12 (6)	2.4 ± 0.60 (5)	4.0 ± 0.94 (6)**	5.2 ± 1.51 (6)**	11 ± 1.20 (5)***	nd
OXA (15, 0.1)	1.0 ± 0.09 (11)	7.1 ± 1.38 (6)***	9.6 ± 2.15 (6)***	3.6 ± 0.92 (6)*	1.2 ± 0.04 (4)	nd
FA (3.6, 10)	1.0 ± 0.05 (6)	0.9 ± 0.11 (6)	1.4 ± 0.31 (5)	1.6 ± 0.41 (5)	0.9 ± 0.32 (5)	nd
MS (30, 25)	1.0 ± 0.12 (6)	0.8 ± 0.10 (6)	1.2 ± 0.18 (6)	1.3 ± 0.20 (6)	1.3 ± 0.21 (6)	nd
Auricular lymph nodes						
TMA (30, 50)	1.0 ± 0.11 (11)	0.8 ± 0.10 (6)	0.7 ± 0.05 (6)	0.8 ± 0.07 (6)	0.5 ± 0.15 (6)	130 ± 22 (3)***
PA (15, 25)	1.0 ± 0.12 (12)	0.8 ± 0.06 (5)	1.6 ± 0.32 (6)	0.9 ± 0.21 (6)	0.8 ± 0.09 (6)	93 ± 21 (3)***
TDI (7.5, 1)	1.0 ± 0.36 (11)	0.6 ± 0.11 (6)	2.0 ± 0.48 (6)*	5.3 ± 0.76 (6)***	15 ± 3.4 (6)***	46 ± 6.3 (3)***
HDI (7.5, 1)	1.0 ± 0.18 (6)	0.9 ± 0.07 (6)	30 ± 6.3 (6)***	96 ± 6.4 (6)***	109 ± 7.7 (6)***	285 ± 18 (6)***
IPDI (7.5, 1)	1.0 ± 0.11 (6)	30 ± 12 (6)***	37 ± 11 (6)***	67 ± 16 (6)***	85 ± 24 (5)***	415 ± 41 (6)***
DNCB (30, 1)	1.0 ± 0.16 (11)	nd	nd	nd	nd	61 ± 3.5 (3)***
OXA (15, 0.1)	1.0 ± 0.1 (10)	3.0 ± 0.8 (6)	1.4 ± 0.41 (6)	26 ± 6.9 (6)***	32 ± 15 (5)***	52 ± 14 (3)***
FA (3.6, 10)	1.0 ± 0.18 (6)	0.9 ± 0.14 (6)	1.2 ± 0.25 (6)	1.6 ± 0.24 (5)	0.8 ± 0.12 (6)	10 ± 1.7 (6)**
MS (30, 25)	1.0 ± 0.20 (6)	1.1 ± 0.12 (6)	0.9 ± 0.07 (5)	1.1 ± 0.20 (6)	0.7 ± 0.16 (6)	2.3 ± 0.2 (3)**

SIs in mandibular and auricular LNs are expressed as mean ± S.E. Statistical analysis was performed after logarithmic transformation, using a one-way ANOVA followed by a two-sided Dunnett's test: $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$.

[a] Within brackets: inhalation exposure concentration in mg/m^3, concentration for skin exposure in % (w/v) (except for FA; 10% (v/v) FA was prepared by adding 8 parts of AOO to 3 parts of FA (37%)).

[b] Number of mice per group.

[c] nd: not done.

utive days. TDI, HDI, and IPDI induced a linear dose–response curve. OXA and DNCB induced a dose–response curve, which reached a plateau already after exposure for 45 min per day for 3 days. In general, respiratory sensitizers induced more IL-4 than contact sensitizers. The highest levels of IL-4 were induced by HDI (10 times higher than DNCB), followed by TDI and TMA (3-fold higher than DNCB) and PA and IPDI (2-fold higher than DNCB).

After skin exposure all respiratory sensitizers induced considerably higher IL-4 responses in the auricular LN when compared to the contact sensitizers DNCB, OXA and FA, while MS did not induce a measurable IL-4 response (Fig. 1b).

3.3. IL-10 induction

Fig. 2a presents the IL-10 induction in the mandibular LN after inhalation exposure. A bell-shaped curve was induced by TMA, PA and IPDI. TDI and HDI induced a dose-dependent curve, while DNCB and OXA did not induce a clear dose–response curve. With the exception of IPDI, which induced lower levels of IL-10 than DNCB and OXA, the respiratory sensitizers induced higher IL-10 levels than did the contact sensitizers DNCB and OXA. TMA induced approximately 3-fold higher levels than DNCB. PA, TDI and HDI induced approximately 2-fold higher levels than DNCB. FA and MS did not induce IL-10 production after inhalation exposure.

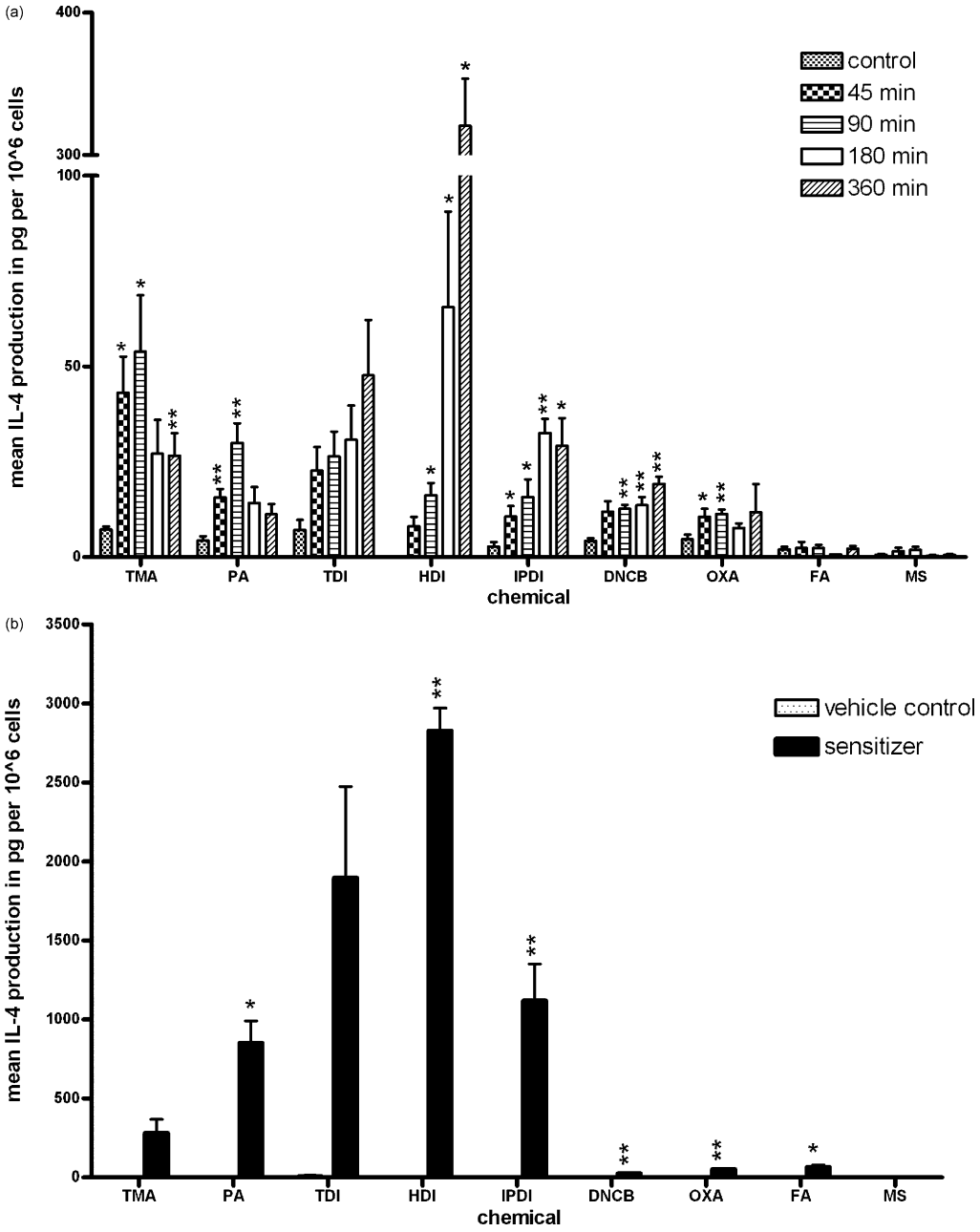

Fig. 1. (a) IL-4 production after inhalation exposure to sensitizers. Mice were exposed to the different chemicals by inhalation exposure for 45, 90, 180 or 360 min per day for 3 consecutive days as described in detail in the methods section. IL-4 production was determined for mandibular LN cells *ex vivo* stimulated with Con A. Data are expressed in pg per 10^6 cells (mean ± S.E.). Asterisks denote significant differences with the vehicle treated mice: $^{*}p < 0.05$ and $^{**}p < 0.01$. (b) IL-4 production after skin exposure to sensitizers. Mice were exposed to the different chemicals by topical exposure 3 consecutive days as described in detail in the methods section. IL-4 production was determined for auricular LN cells *ex vivo* stimulated with Con A. Data are expressed in pg per 10^6 cells (mean ± S.E.). Asterisks denote significant differences with the vehicle treated mice: $^{*}p < 0.05$ and $^{**}p < 0.01$.

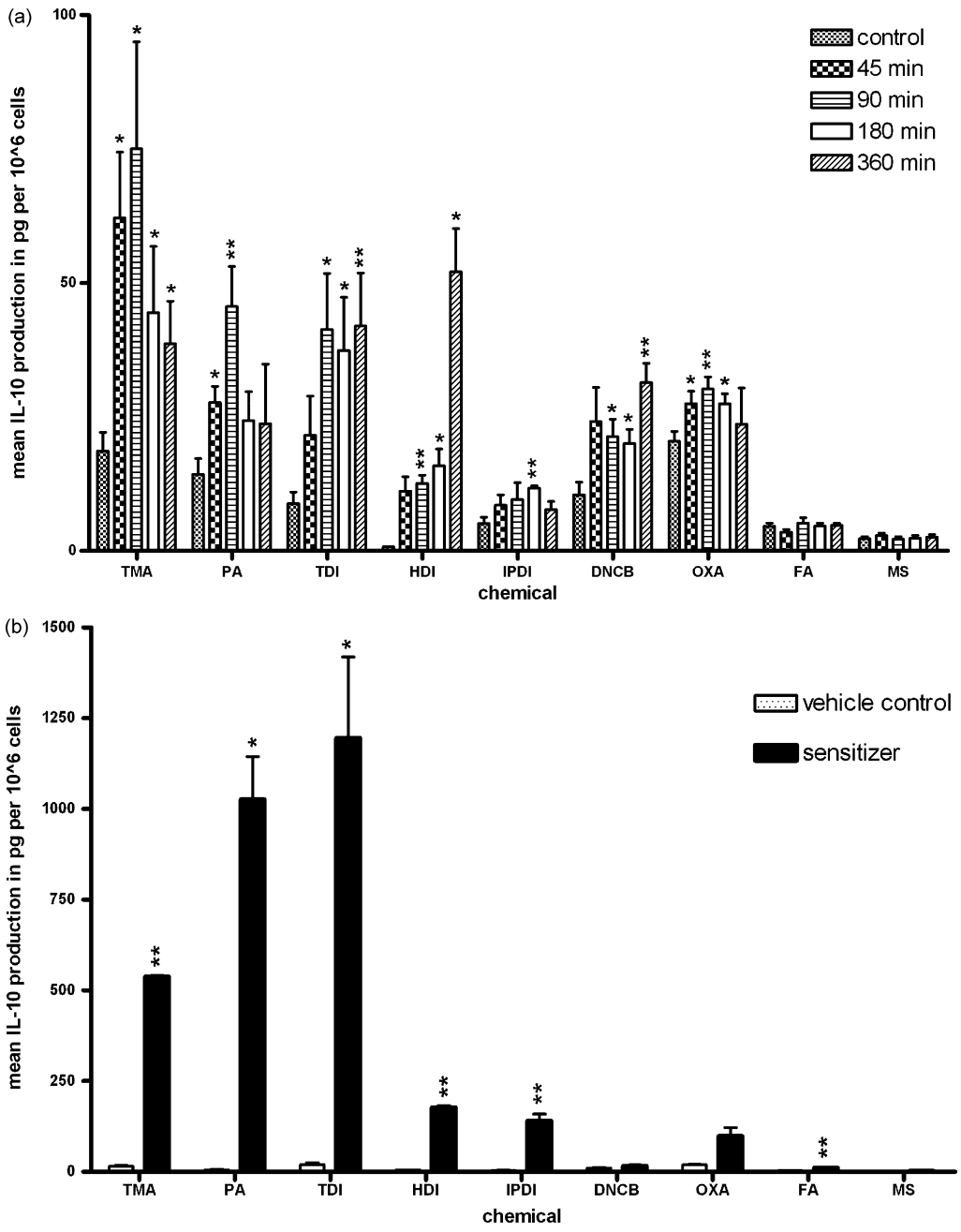

Fig. 2. (a) IL-10 production after inhalation exposure to sensitizers. Mice were exposed to the different chemicals by inhalation exposure for 45, 90, 180 or 360 min per day for 3 consecutive days as described in detail in the methods section. IL-10 production was determined for mandibular LN cells *ex vivo* stimulated with Con A. Data are expressed in pg per 10^6 cells (mean ± S.E.). Asterisks denote significant differences with the vehicle treated mice: $^{*}p < 0.05$ and $^{**}p < 0.01$. (b) IL-10 production after skin exposure to sensitizers. Mice were exposed to the different chemicals by topical exposure 3 consecutive days as described in detail in the methods section. IL-10 production was determined for auricular LN cells *ex vivo* stimulated with Con A. Data are expressed in pg per 10^6 cells (mean ± S.E.). Asterisks denote significant differences with the vehicle treated mice: $^{*}p < 0.05$ and $^{**}p < 0.01$.

After skin exposure, high levels of IL-10 were induced especially by TMA, PA and TDI. HDI, IPDI and OXA induced lower levels, whereas DNCB and FA induced only very low levels of IL-10 (Fig. 2b).

3.4. IFN-γ induction

Fig. 3a presents the induction of IFN-γ in the mandibular LN after inhalation exposure. The highest induction was found for the contact sensitizers DNCB and OXA. The response induced by DNCB was clearly dose-dependent. TMA, TDI, and OXA induced a bell-shaped curve, whereas no dose–response was found after exposure to PA, HDI and IPDI. When compared to the highest response induced by the skin sensitizer DNCB, the respiratory sensitizers TMA, TDI and HDI induced approximately 4 times less IFN-γ. DNCB induced approximately 10-fold higher levels of IFN-γ than PA and IPDI. The contact sensitizer FA induced only low levels of IFN-γ and the irritant MS did not induce IFN-γ production after inhalation exposure.

After skin exposure IFN-γ was induced by both the respiratory sensitizers and the contact sensitizers DNCB and OXA (Fig. 3b). The highest levels were induced by TMA, followed by HDI, TDI and DNCB. The LMW chemicals PA, IPDI and OXA induced lower levels of IFN-γ, whereas FA induced only low, but detectable levels of IFN-γ and MS did not induce IFN-γ at all.

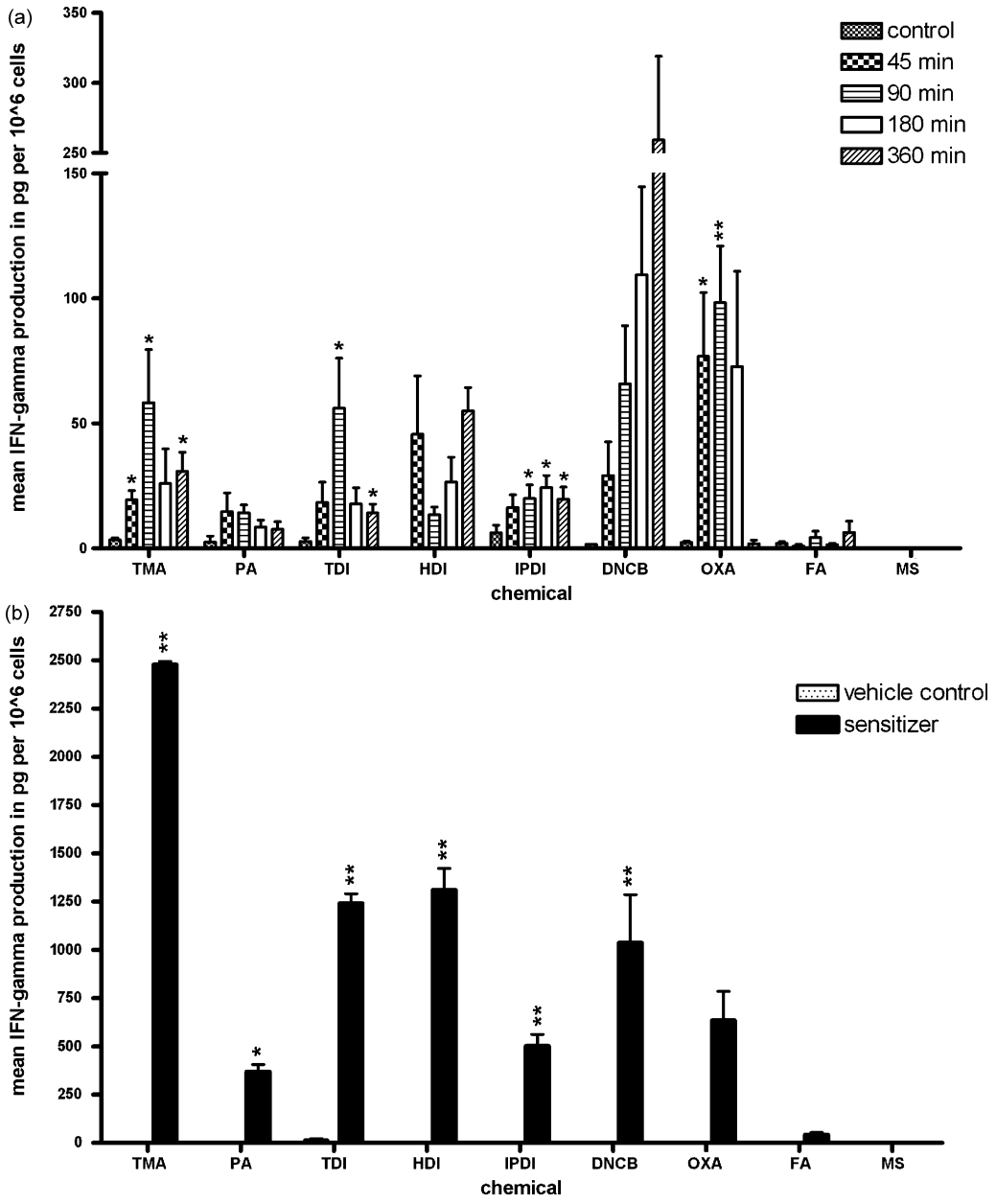

Fig. 3. (a) IFN-γ production after inhalation exposure to sensitizers. Mice were exposed to the different chemicals by inhalation exposure for 45, 90, 180 or 360 min per day for 3 consecutive days as described in detail in the methods section. IFN-γ production was determined for mandibular LN cells *ex vivo* stimulated with Con A. Data are expressed in pg per 10^6 cells (mean ± S.E.). Asterisks denote significant differences with the vehicle treated mice: $^{*}p < 0.05$ and $^{**}p < 0.01$. (b) Relative IFN-γ production after skin exposure to sensitizers. Mice were exposed to the different chemicals by topical exposure 3 consecutive days as described in detail in the methods section. IFN-γ production was determined for auricular LN cells *ex vivo* stimulated with Con A. Data are expressed in pg per 10^6 cells (mean ± S.E.). Asterisks denote significant differences with the vehicle treated mice: $^{*}p < 0.05$ and $^{**}p < 0.01$.

3.5. IL-12 induction

Both respiratory and contact sensitizers induced low levels of IL-12 upon inhalation exposure; all test compounds, except FA and MS, induced comparable levels (between 5 and 20 pg per 10^6 cells). The levels of IL-12 that were induced by contact sensitizers were not different from those induced by respiratory sensitizers. FA and MS induced low levels of IL-12 (<5 pg per 10^6 cells; data not shown.).

3.6. IL-4 and IFN-γ production as a function of the proliferative response

IL-4 and IFN-γ levels were plotted as a function of the SI values (Fig. 4). For some chemicals the dose–response curves of proliferative and cytokine responses were bell-shaped and decreased with increasing duration of exposure. Therefore, some data points were excluded from the plots. For TMA, PA, and OXA the time-points 180 and 360 min/day and for TDI and IPDI the time-point 360 min/day were not used in Fig. 4.

At a low SI (=2), IL-4 production (Fig. 4a), can only be used to identify TMA. However, at intermediate SI values (4–7), all respiratory sensitizers, except HDI, induced higher levels of IL-4 than the contact sensitizers. When the respiratory sensitizers were compared with DNCB at these SI values, the amounts were 4.7-fold higher for TMA ($p = 0.0015$), 2.1-fold for PA (not significant), 2.4-fold for TDI ($p = 0.043$) and 2.5-fold for IPDI ($p = 0.001$). All respiratory sensitizers except HDI induced significantly higher levels IL-4 than OXA. At these SI values the amounts were 5-fold higher for TMA

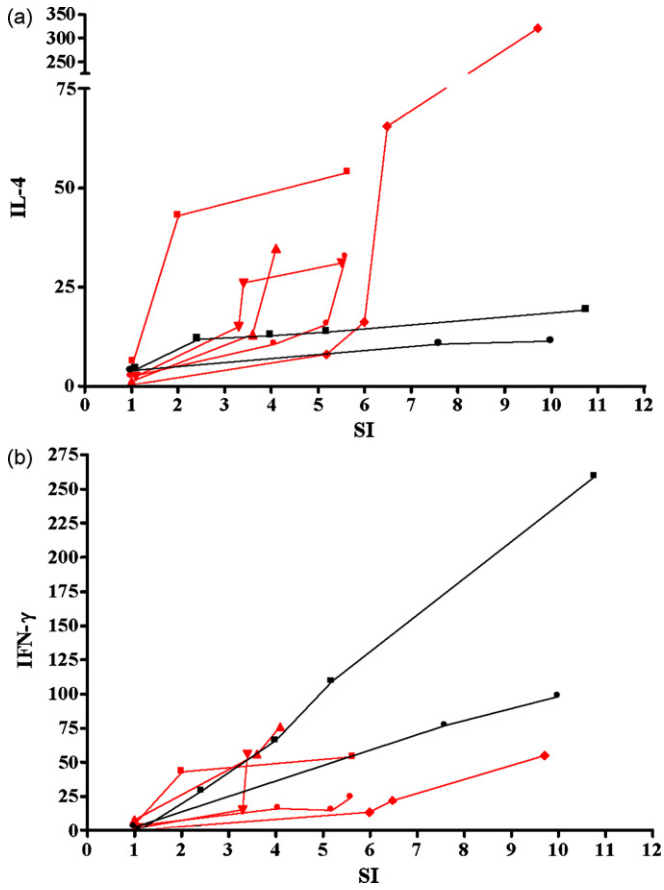

Fig. 4. IL-4 (a) and IFN-γ (b) are plotted as a function of the stimulation index (SI) for the following respiratory sensitizers (in red): TMA (■), PA (▲), TDI (▼), HDI (♦), IPDI (●) and contact sensitizers (in black): DNCB (■) and OXA (●).

($p = 0.005$), 2.6-fold for PA ($p = 0.0041$), 2.9-fold for TDI ($p = 0.013$) and 3.1-fold for IPDI ($p = 0.0005$). High SI values (>6) were only induced by HDI, DNCB and OXA (Table 1). The IL-4 production of HDI showed a steep increase after SI = 6, this was not observed for DNCB and OXA, which reached a plateau at SI = 2 and SI = 7.6, respectively. At an SI = 10, significantly higher IL-4 levels were measured after HDI exposure than after DNCB (20-fold increase, $p = 0.00016$) and OXA exposure (35-fold, $p = 0.00028$).

In Fig. 4b, IFN-γ is plotted as a function of the SI values. At SI values below 6 it was not possible to distinguish respiratory from skin sensitizers at SI values <6. At an SI value of 10, the highest levels of IFN-γ were induced by DNCB, followed by OXA and HDI. IFN-γ production after HDI exposure was significantly lower than after DNCB (4.7-fold, $p = 0.0025$) and OXA (1.5-fold, $p = 0.0049$) exposure.

IL-10 was also plotted against the SI values, but this plot could not be used to discriminate between contact and respiratory sensitizers (data not shown).

4. Discussion

In a LLNA, using inhalation instead of skin exposure, a selection of contact and respiratory sensitizers was investigated. All contact and respiratory sensitizers, except FA, were identified in this so-called respiratory LLNA, by measuring an increase in lymphocyte proliferation in the mandibular LNs (Arts et al., 2008). In addition, assessment of cytokines provided information that enabled discrimination between respiratory and contact sensitizers. In the respiratory LLNA it was shown that after inhalation exposure to respiratory sensitizers relatively high levels of IL-4 and low levels of IFN-γ were produced than after inhalation exposure to contact sensitizers. The production of IL-10 might add additional information, but appears to be less suitable for the identification of respiratory sensitizers. IL-12 production was low and could not be used to distinguish between contact and respiratory sensitizers.

These cytokine responses are in line with those obtained after dermal exposure and intranasal application (Betts et al., 2002; Dearman et al., 2002; Farraj et al., 2006, 2007; Plitnick et al., 2002; Vandebriel et al., 2000, 2003; Vanoirbeek, 2006). The amount of cytokines that was produced was for almost all chemicals orders of magnitude higher after skin exposure than after inhalation exposure. However, the skin LLNA was included as a positive control in these tests and only one high concentration of each compound was used, resulting in very high SI values (Table 1).

Van Och et al. (2002) have shown in the skin LLNA that there is a linkage between proliferation and cytokine profiles. The identification of respiratory sensitizers based on IL-4 production was possible at concentration levels inducing relatively low SI values. In contrast, contact sensitizers could only be identified by IFN-γ at concentration levels inducing very high stimulation indices (SI values ≥35). In the respiratory LLNA, it was not possible to achieve such high stimulation indices, because high exposure concentrations resulted in epithelial damage, mainly in the upper respiratory tract (Arts et al., 2008). Although in the respiratory LLNA SI values were much lower than in the skin LLNA, a similar trend as described by Van Och et al. (2002) was observed. IL-4 production could be used to identify all respiratory sensitizers, except HDI, at intermediate SI values (SI = 4–6), whereas IFN-γ was only discriminative for contact sensitizers at higher SI values (SI = 10), which were only induced by DNCB, OXA and HDI.

One of the advantages of the modified LLNA is that proliferation and cytokine profiling can be performed in the same animal, thus offering an opportunity to reduce the number of animals which is needed in separate LLNA and cytokine assays. In addition, it requires much less radioactive label and therefore waste. The added value of the inhalation model is the possibility to determine the potency of LMW chemicals to induce an immune response in the respiratory tract possibly leading to sensitization. Indeed, the potency ranking differed after inhalation exposure from that in the skin LLNA with the typical respiratory sensitizers, viz. diisocyanates and acid anhydrides being more potent than DNCB after inhalation on a calculated mean dose per animal (Arts et al., 2008).

In this study it is demonstrated that respiratory and contact sensitizers can sensitize the body via dermal as well as respiratory exposure and that the route of exposure did not affect the cytokine profiles induced by the sensitizers. Hence, contact and respiratory sensitizers possess the intrinsic ability to polarize the immune response to Th1 or Th2, respectively. In this respect it is important to note that LMW chemicals can induce not only type I immune responses in the lungs, leading to allergic asthma (involving the conducting airways), but also type IV immune responses, i.e. extrinsic allergic alveolitis or hypersensitivity pneumonitis (Farraj et al., 2004; O'Hollaran, 1995; Sala et al., 1996). For example, the respiratory allergen TMA has been associated with allergic alveolitis in workers exposed to high concentrations (Zeiss and Patterson, 1993). The association of induction of a type IV immune response due to exposure to high concentrations of TMA is corroborated by the low levels of IL-4 and the high levels of IFN-γ induced upon the high dermal dose. After inhalation, extended exposure to TMA does not skew the cytokine profile towards Th1; a bell-shaped cytokine dose–response profile was found for both IL-4 and IFN-γ. Studies with DNCB and typical contact allergens such as DNFB and picryl chloride (trinitrochlorobenzene) using dermal sensitization

followed by inhalation, intranasal or intratracheal challenge have also shown the potential of contact senzitizers to elicit hypersensitivity reactions in the respiratory tract (Arts et al., 1998; Buckley and Nijkamp, 1994; Garssen et al., 1989, 1994; Satoh et al., 1995; Vanoirbeek et al., 2006; Farraj et al., 2007). As such, contact sensitizers may pose a risk to human health when inhalation exposure is likely to occur.

Overall, after inhalation exposure induction of IL-4 could be used to identify respiratory sensitizers both at intermediate and high SI values, whereas the contact sensitizers could only be identified when IFN-γ was measured at high stimulation indices. Following skin application, it appeared that IL-4 and IL-10 could also be used for the identification of respiratory sensitizers, whereas IFN-γ levels were not discriminative between respiratory and contact sensitizers. In conclusion, the respiratory LLNA is able to identify and distinguish strong contact and respiratory sensitizers when simultaneously proliferation and cytokine production are assessed in the upper respiratory tract draining LNs.

Conflict of interest statement

All authors declare that there is no conflict of interest.

Acknowledgements

The authors thank the following persons for their invaluable help when performing the studies: Evert Duistermaat, Liset De La Fonteyne, Eric Gremmer, Frank Hendriksma, Piet van de Heuvel, Gerard Roverts, Piet van Schaaik, Hans Strootman, Henny Verharen and Bert Verlaan. This research was funded by the Dutch Ministry of Social Affairs and Employement (SZW), the Dutch Ministry of Health, Welfare and Sports (VWS) and the Dutch Food and Consumer Safety Authority (VWA).

References

Aronica, M.A., Mora, A.L., Mitchell, D.B., Finn, P.W., Johnson, J.E., Sheller, J.R., Boothby, M.R., 1999. Preferential role for NF-kB/Rel signalling in type 1 but not type 2 T cell dependent immune response in vivo. J. Immunol. 163, 5116–5124.

Arts, J.H.E., 2001. Respiratory Allergy Induced by Low Molecular Weight Chemicals in Rats. PhD Thesis. Utrecht University, Utrecht, The Netherlands.

Arts, J.H.E., De Jong, W.H., Van Triel, J.J., Schijf, M.A., De Klerk, A., Van Loveren, H., Kuper, F., 2008. The respiratory local lymph node assay (LLNA) as a tool to study respiratory sensitizers. Toxicol. Sci. 106, 423–434.

Arts, J.H.E., Kuper, C.F., 2007. Animal models to test respiratory allergy of low molecular weight chemicals: a guidance. Methods 41, 61–71.

Arts, J.H.E., Kuper, C.F., Spoor, S.M., Bloksma, N., 1998. Airway morphology and function of rats following dermal sensitization and respiratory challenge with low molecular weight chemicals. Toxicol Appl. Pharmacol. 152, 66–76.

Basketter, D.A., Lea, L.J., Cooper, K., Stocks, J., Dickens, A., Pate, I., Dearman, R.J., Kimber, I., 1999a. Threshold for classification as a skin sensitizer in the local lymph node assay: a statistical evaluation. Food Chem. Toxicol. 37, 1167–1174.

Basketter, D.A., Lea, L.J., Dickens, A., Briggs, D., Pate, I., Dearman, R.J., Kimber, I., 1999b. A comparison of statistical approaches to the derivation of EC3 values from local lymph node assay dose responses. J. Appl. Toxicol. 19, 261–266.

Betts, C.J., Dearman, R.J., Flanagan, B.F., Kimber, I., 2002. Temporal changes in cytokine gene expression profiles induced in mice by trimellitic anhydride. Toxicol. Lett. 136, 121–132.

Buckley, T.L., Nijkamp, F.P., 1994. Mucosal exudation associated with pulmonary delayed type hypersensitivity reaction in the mouse. Role for tachykinins. J. Immunol. 153, 4169–4178.

Dearman, R.J., Basketter, D.A., Kimber, I., 1995. Differential cytokine production following chronic exposure of mice to chemical respiratory and contact allergens. Immunology 86, 545–550.

Dearman, R.J., Basketter, D.A., Kimber, I., 1996. Characterization of chemical allergens as a function of divergent cytokine secretion profiles induced in mice. Toxicol. Appl. Pharmacol. 138, 308–316.

Dearman, R.J., Hilton, J., Basketter, D.A., Kimber, I., 1999. Cytokine endpoints for the local lymph node assay: consideration of interferon gamma and interleukin 12. J. Appl. Toxicol. 19, 149–155.

Dearman, R.J., Warbrick, E.V., Skinner, R., Kimber, I., 2002. Cytokine fingerprinting of chemical allergens: species comparison and statistical analysis. Food Chem. Toxicol. 40, 1881–1892.

De Jong, W.H., Van Och, F.M.M., Den Hartog Jager, C.F., Spiekstra, S.W., Slob, W., Vandebriel, R., Van Loveren, H., 2002. Ranking of allergenic potency of rubber chemicals in a modified local lymph node assay. Toxicol. Sci. 66, 226–232.

EC, 2006. Regulation (EC) No. 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No. 793/93 and Commission Regulation (EC) No. 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. O. J. Eur. Commun. (L396), 1–849 (http://ec.europa.eu/environmental/chemicals/reach/reach_intro.html).

Farraj, A.K., Boykin, E., Haykal-Coates, N., Gavett, S.H., Doerfler, D., Selgrade, M., 2007. Th2 cytokines in skin draining lymph nodes and serum IgE do not predict airway hypersensitivity to intranasal isocyanate exposure in mice. Toxicol. Sci. 100, 99–108.

Farraj, A.K., Harkema, J.R., Kaminski, N.E., 2004. Allergic rhinitis induced by intranasal sensitization and challenge with trimellitic anhydride but not with dinitrochlorobenzene or oxazolone in A/J mice. Toxicol. Sci. 79, 315–325.

Farraj, A.K., Harkema, J.R., Kaminski, N.E., 2006. Topical application versus intranasal instillation: a qualitative comparison of the effect of route of sensitization on trimellitic anhydride-induced allergic rhinitis in A/J mice. Toxicol. Sci. 92, 321–328.

Garssen, J., Nijkamp, F.P., Wagenaar, S.Sc., Zwart, A., Askenase, P.W., Van Loveren, H., 1989. Regulation of delayed type hypersensitivity responses in the mouse lung, determined with histological procedures: serotonin, T-cell supressor-inducer factor and high antigen dose tolerance regulate the magnitude of T-cell dependent inflammatory reactions. Immunology 68, 51–58.

Garssen, J., Nijkamp, F.P., Van Der Vliet, H., Van Loveren, H., 1994. A role for cellular immunity in the induction of airway hyperresponsiveness induced by small molecular weight compounds. Toxicol. Lett. 72, 151–154.

Haneke, K.E., Tice, R.R., Carson, B.L., Margolin, B.H., Stokes, W.S., 2001. ICCVAM evaluation of the murine local lymph node assay. III. Data analysis completed by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods. Regul. Toxicol. Pharmacol. 34, 274–286.

Kimber, I., Basketter, D.A., 1992. The murine local lymph node assay: a commentary on collaborative studies and new directions. Food Chem. Toxicol. 30, 165–169.

Kimber, I., Agius, R., Basketter, D.A., Corsini, E., Cullinan, P., Dearman, R.J., Gimenez-Arnau, E., Greenwell, L., Hartung, T., Kuper, F., Maestrelli, P., Roggen, E., Rovida, C., 2007. Chemical respiratory allergy: opportunities for hazard identification and characterisation. The report and recommendations of ECVAM workshop 60. Altern Lab Anim 35, 243–265.

Kimber, I., Weisenberger, C., 1989. A murine local lymph node assay for the identification of contact allergens. Assay development and results of an initial validation study. Arch. Toxicol. 63, 274–282.

OECD, 2002. Guideline 429. Guideline for the Testing of Chemicals. Skin Sensitisation: Local Lymph Node Assay. OECD, Paris, France.

O'Hollaran, M.T., 1995. Dyspneu and larynx. Ann. Allergy Asthma Immunol. 75, 1–4.

Piccotti, J.R., Kawabata, T.T., 2008. Use of an ex vivo local lymph node assay to assess contact hypersensitivity potential. J. Immunotoxicol. 5, 271–277.

Piccotti, J.R., Knight, S.A., Gillhouse, K., Lagattuta, M.S., Bleavins, M.R., 2006. Evaluation of an ex vivo murine local lymph node assay: multiple endpoint comparison. J. Appl. Toxicol. 26, 333–340.

Plitnick, L.M., Loveless, S.E., Ladics, G.S., Holsapple, M.P., Selgrade, M.J., Sailstad, D.M., Smialowicz, R.J., 2002. Cytokine profiling for chemical sensitizers: application of the ribonuclease protection assay and effect of dose. Toxicol. Appl. Pharmacol. 179, 145–154.

Sala, E., Hytönen, M., Tupasela, O., Estlander, T., 1996. Occupational laryngitis with immediate allergic or immediate type specific chemical hypersensitivity. Clin. Otolaryngol. Allied Sci. 21, 42–48.

Satoh, T., Kramarik, J.A., Tollerud, D.J., Karol, M.H., 1995. A murine model for assessing the respiratory hypersensitivity potential of chemical allergens. Toxicol. Lett. 78, 57–66.

Vandebriel, R.J., De Jong, W.H., Hendriks, J.J.A., Van Loveren, H., 2003. Impact of exposure duration by low molecular weight compounds on interferon-γ and interleukin-4 mRNA expression and production in the draining lymph nodes of mice. Toxicology 188, 1–13.

Vandebriel, R.J., De Jong, W.H., Spiekstra, S.W., Van Dijk, M., Fluitman, A., Garssen, J., Van Loveren, H., 2000. Assessment of preferential T-Helper 1 or T-Helper 2 induction by low molecular weight compounds using the local lymph node assay in conjunction with RT-PCR and ELISA for interferon-gamma and interleukin-4. Toxicol. Appl. Pharmacol. 162, 77–85.

Van Loveren, H., Cockshott, A., Gebel, T., Gundert-Remy, U., De Jong, W.H., Matheson, J., McGarry, H., Musset, L., Selgrade, M.K., Vickers, C., 2008. Skin sensitization in chemical risk assessment: report of a WHO/IPCS international workshop focusing on dose–response assessment. Regul. Toxicol. Pharmacol. 50, 155–199.

Van Och, F.M.M., Van Loveren, H., De Jong, W.H., Vandebriel, R., 2002. Cytokine production induced by low molecular weight chemicals as a function of the stimulation index in a modified local lymph node assay: an approach to discriminate contact sensitizers from respiratory sensitizers. Toxicol. Appl. Pharmacol. 184, 46–56.

Van Och, F.M.M., Slob, W., De Jong, W.H., Vandebriel, R., Van Loveren, H., 2000. A quantitative method for assessing the sensitizing potency of low molecular weight chemicals using a local lymph node assay: employment of a regression

method that includes determination of the uncertainty margins. Toxicology 146, 49–59.
Vanoirbeek, J.A., Tarkowski, M., Vanhooren, H.M., De Vooght, V., Nemery, B., Hoet, P., 2006. Validation of a mouse model of chemical induced asthma using trimellitic anhydride, a respiratory sensitizer, and dinitrochlorobenzene, a dermal sensitizer. J. Allergy Clin. Immunol. 117, 1090–1097.
Woolhiser, M.R., Munson, A.E., Meade, B.J., 2000. Comparison of mouse strains using the local lymph node assay. Toxicology 146, 221–227.
Zeiss, C.R., Patterson, R., 1993. Acid anhydrides. In: Bernstein, I.L., Chan-Yeung, M., Malo, J.-L., Bernstein, D.I. (Eds.), Asthma in the Workplace, 1st ed. Marcel Dekker, New York, pp. 439–457.