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Identification of contact and respiratory sensitizers according to IL-4 receptor α expression and IL-2 production

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ABSTRACT

Identification of allergenic chemicals is an important occupational safety issue. While several methods exist to identify contact sensitizers, there is currently no validated model to predict the potential of chemicals to act as respiratory sensitizers. Previously, we reported that cytometry analysis of the local immune responses induced in mice dermally exposed to the respiratory sensitizer trimellitic anhydride (TMA 10%) and contact sensitizer dinitrochlorobenzene (DNCB 1%) could identify divergent expression of several immune parameters. The present study confirms, first, that IgE-positive B cells, MHC class II molecules, interleukin (IL)-2, IL-4 and IL-4R α can differentiate the allergic reactions caused by high doses of strong respiratory (TMA, phthalic anhydride and toluene diisocyanate) and contact sensitizers (DNCB, dinitrofluorobenzene and oxazolone). The second part of the study was designed to test the robustness of these markers when classing the weakly immunogenic chemicals most often encountered. Six respiratory allergens, including TMA (2.5%), five contact allergens, including DNCB (0.25%), and two irritants were compared at doses of equivalent immunogenicity. The results indicated that IL-4R α and IL-2 can be reliably used to discriminate sensitizers. Respiratory sensitizers induced markedly higher IL-4R α levels than contact allergens, while irritants had no effect on this parameter. Inversely, contact allergens tended to induce higher percentages of IL-2⁺CD8⁺ cells than respiratory allergens. In contrast, the markers MHC-II, IgE and IL-4 were not able to classify chemicals with low immunogenic potential. In conclusion, IL-4R α and IL-2 have the potential to be used in classifying a variety of chemical allergens.

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Introduction

Exposure to low molecular weight (LMW) chemicals present in the work environment is an important cause of occupational hypersensitivity pathologies (Mapp et al., 2005). Among the various chemicals found in the industrial sector, a wide range of substances, such as metal salts (Bright et al., 1997; Fernandez-Nieto et al., 2006), fragrance ingredients (Rastogi et al., 2001; Trattner and David, 2004) or halogenated aromatic compounds (Ashby et al., 1995) are known for their capacity to induce allergic contact dermatitis (ACD), a T-cell mediated delayed-type hypersensitivity reaction. Compared to the large number of known contact sensitizers, the list of chemicals known to cause respiratory allergy, generally an IgE, Th2-mediated response, is shorter. In industrial countries however, the LMW chemicals such as acid anhydrides (Bernstein, 2003; Zeiss, 2002) and isocyanates (Liu and Wisniewski, 2003), are involved in 40% of the occupational asthma cases. Moreover, they represent a major risk for

workers since asthma does lead over time to lung damage which can also ultimately be fatal. Thus, clear identification and characterization of chemicals capable of eliciting either respiratory or dermal allergies is an important issue in occupational safety, and newly synthesized molecules need to be assessed before being handled or inhaled by workers in order to adopt preventive measures.

A great majority of chemical allergens have physicochemical and electrophilic properties allowing them to penetrate the skin or the tracheal membrane. They can then form covalent bonds with nucleophilic proteins encountered in the epidermal or bronchial mucosa. This reaction, which occurs either directly or following chemical activation through endogenous metabolism, generates an immunogenic complex which can then trigger an immune response (Lepoittevin, 2005). Hence, computational studies, based on the quantitative structure–activity relationships (QSARs) of the LMW compounds, contribute to the prediction of allergic contact dermatitis (Patlewicz et al., 2004) and occupational asthma hazards (Jarvis et al., 2005; Seed and Agius, 2010). Currently, however, this molecular approach can only highlight the potential of chemicals to be sensitizers. Their true effect must be confirmed by *in vivo* or *in vitro* methods.

While validated animal methods such as the local lymph node assay (LLNA) (OECD, 2001), and newly developed *in vitro* tests can be used, or are going to be used, to identify the potential contact

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allergenicity of chemicals, there is currently no model to prospectively identify chemicals inducing allergic asthma. Although the LLNA involves skin contact, it has also been shown to be applicable for respiratory allergens (Kimber et al., 2007; Arts et al., 2008). However, the results of the LLNA indicate whether an immune response has been triggered, but they cannot distinguish the specific mechanisms inherent to respiratory or contact allergy (Boverhof et al., 2008; Gerberick et al., 2005; Vanoirbeek et al., 2003). Humoral and cellular immune responses develop according to different mechanisms that depend mainly on the specific activation and expansion of CD4⁺ T helper (Th) cells into different subsets such as Th1, Th2, Th17 and regulatory T cells (Treg) with their own cytokine signatures (Adkis, 2008). Although all of these T cell populations have the potential to influence hypersensitivity responses, this paper focuses on the dichotomy of Th1 and Th2. Th1 cells which essentially produce interleukin-2 (IL-2) and interferon- γ (IFN- γ) play a pivotal role in ACD, while Th2 cells produce IL-4, IL-5 and IL-13 and control the development of IgE-mediated hypersensitivity reactions such as asthma (Mosmann and Sad, 1996). Similarly, the CD8⁺ T cytotoxic (Tc) cells differentiate into Tc1 and Tc2 subsets, each characterized by specific cytokine profiles (Woodland and Dutton, 2003). The specific pattern of cytokines produced in the early stages of sensitization determines the nature of the immune response. So far, studies to identify immune markers differentiating LLNA-positive-chemicals have mainly used *in vivo* methods. Indeed, respiratory allergens still require animal assays, as most of them are not water soluble and thus cannot be used in cell-based assays. The main parameters investigated were, serum IgE levels (Ban and Hettich, 2001; Guo et al., 2002; Hilton et al., 1996; Manetz and Meade, 1999) and cytokine production by draining lymph nodes cells (Farraj et al., 2006; Manetz et al., 2001; Plitnick et al., 2002, 2005; Sailstad et al., 2003). However, due to a lack of consensus, these assays have not yet allowed discriminating parameters to be defined.

We previously reported that cytometry analysis of the local immune responses induced in mice after dermal exposure to reference respiratory and contact sensitizers (trimellitic anhydride (TMA) and dinitrochlorobenzene (DNCB), respectively) could identify divergent expression of several immune factors (Goutet et al., 2005). Thus, increased proportion of IgE⁺ cells and enhanced expression of MHC II molecules were observed in lymph node B lymphocytes from TMA-treated mice. At the same time, the proportion of IL-4 producing CD4⁺ lymphocytes and IL-4 receptor (CD124) expression, were also clearly higher. In contrast, in DNCB-treated mice, higher proportions of IL-2 producing cells were detected in CD4⁺ and CD8⁺ subsets. To ensure the relevance of these parameters in a risk-assessment setting, it was necessary to study the allergenic behavior of a wider range of LMW sensitizers.

In the first part of the present study, we checked that other strong respiratory and contact sensitizers induced the same specific immune responses as TMA and DNCB in mice. In the second part, we investigated a panel of various chemical allergens and irritants so as to identify the most pertinent and reliable markers to classify chemicals with different immunogenic potential.

Materials and methods

Animals. All experiments were carried out with female BALB/c mice (8–10 weeks old) obtained from Charles River (Italy). Mice were allowed to acclimatize for 1 week to laboratory conditions, at 21 $\pm$ 1 °C, before treatment and were provided with a standard diet and fresh water *ad libitum*.

Chemicals. 1,2,4-benzenetricarboxylic (trimellitic) anhydride (TMA) 97%, phthalic anhydride (PA), 4,4'-methylene-bis-phenylisocyanate (MDI), p-tolylmonoisoocyanate (TMI), sodium hexachloroplatinate (Pt), all purchased from Sigma-Aldrich (Steinheim, Germany) and

toluene 2,4-diisocyanate (TDI) from Merck (Darmstadt, Germany) were selected as respiratory allergens. 1-chloro-2,4-dinitrobenzene (DNCB) 99%, eugenol (Eug) 99%, nickel sulfate (Ni), α -hexylcinnamaldehyde (HCA) 85%, 4-ethoxymethylene-2-phenyloxazol-5-one (OXA), 1-fluoro-2,4-dinitrobenzene (DNFB), all from Sigma-Aldrich and potassium bichromate (Cr) from Merck were selected as contact allergens. With the exception of TMI, all these chemicals were chosen from a panel of respiratory and skin sensitizers listed by ECETOC (European Center for Ecotoxicology and Toxicology of Chemicals) as reference products to assess new methods of skin sensitization testing (ECETOC, 1999). TMI, is suspected but not proven to induce asthma (Plitnick et al., 2005). Sodium dodecyl sulfate (SDS) and 4-aminobenzoic acid (pABA) (Sigma-Aldrich) were selected as irritants. Acetone 99.7% purex analytical grade was obtained from SDS (Peypin, France), olive oil from Fluka (Saint Quentin Fallavier, France) and dimethyl sulfoxide (DMSO) 99.5% from Sigma-Aldrich. SDS and the three metal salts were dissolved in a DMSO:water mixture (8.5:1.5 v/v), the other chemicals were dissolved in a mixture of acetone:olive oil (AOO) (4:1 v/v). Test solutions were freshly prepared immediately prior to application.

Experimental design. Two sets of chemicals were studied separately. Chemicals in the first set had high immunogenic potential, while chemicals in the second group had lower potential. TMA and DNCB were used in both sets as reference respiratory and contact sensitizers, respectively. The sensitizing concentrations used in the first set, 10% TMA and 1% DNCB, were selected as doses immunologically equivalent according to previous studies (Dearman et al., 1997; Goutet et al., 2005; Van Och et al., 2000). The TMA and DNCB concentrations used in the second set were respectively 2.5% and 0.25% DNCB. In each set, the chemical doses were adjusted to be equally immunogenic. This was determined using a LLNA-modified assay, in which the cell number in lymph nodes was considered as an indicator of proliferation (Goutet et al., 2005). Briefly, chemicals or vehicle were applied daily to the dorsum of both ears in 25 μ l doses to groups of mice (n = 4) for 3 consecutive days. The auricular lymph nodes were excised 72 h following the last application, pooled for each animal, and cells were enumerated using a Coulter Z1 counter. With the nickel salt, two successive applications at a 2-h interval were necessary to induce cellular proliferation. To standardize the application protocol, the other water soluble chemicals were also applied in 2 daily applications.

The same protocol was used to expose mice in the two experiments. On days 0 and 5, mice (four per group) were exposed bilaterally on each shaved flank to chemical solutions (50 μ l of the AOO solutions or 2 $\times$ 50 μ l of the DMSO solutions). On days 10, 11 and 12, animals received challenge doses of 25 μ l or 2 $\times$ 25 μ l of the same chemicals or vehicles on the dorsum of both ears. In the first set study, mice were exposed to one sensitizing concentration (C) of each chemical and three challenge concentrations C, C/2 or C/4. In the second set, the same concentration was used to sensitize and challenge mice. Control groups were exposed to vehicle alone. On day 13, mice were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg, Sanofi Sante Animale, France), and sera in addition to auricular lymph nodes were collected.

Monoclonal antibodies. The following monoclonal antibodies (mAbs) and their matched isotype controls were obtained from BD-Pharmingen (Pont-de-Claix, France): anti-CD3e-CyTM5 (clone 145-2C11), anti-CD4-CyTM5 (clone H129.19), anti-CD8a-fluorescein-isothiocyanate (FITC) (clone 53-6.7), anti-CD19-R-Phycoerythrin (PE) (clone 1D3), anti-CD124-PE (clone Mil 4R-M1), anti-IgE-FITC (clone R35-72), anti-I-A^d-FITC (clone AMS-32.1), anti-CD86 (clone GL1), anti-CD16/CD32 (FcBlock) (clone 2.4G2), anti-IL-2-PE (clone JES6-5H4), anti-IL-4-PE (clone BVD4-1D11), anti-IFN- γ -PE (clone XMG1.2). All mAbs to mouse cell surface antigens or intracellular cytokines were used at saturating concentrations.

Total serum IgE levels. Blood samples were allowed to clot for 4 h, and then centrifuged at 3000 rpm. Serum fractions were collected and stored at -30°C until analysis. Total serum IgE levels were determined using the Mouse IgE OptEIA™ kit (BD Biosciences, San Diego, USA) according to the manufacturer's protocol.

Preparation of auricular lymph node cells. Single cell suspensions were prepared from lymph node pairs of individual animals by mechanical tissue disaggregation through a sterile $40\text{ }\mu\text{m}$ cell strainer (Falcon) in cold RPMI-1640 medium containing 25 mM Hepes (Gibco-Life Technologies) and 1% heat-inactivated fetal calf serum (FCS) (Biosys, France). The number of auricular lymph node cells per animal was determined using a Coulter Z1 counter. After washing, cell pellets were resuspended at 5×10^6 cells/ml in RPMI-1640, 25 mM Hepes, 10% FCS (Biosys), 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (all from Gibco-LT).

Cell surface staining. Cell surface staining was performed on fresh cells with mAbs in combinations of three colors. Approximately 5×10^5 lymph node cells in supplemented RPMI were distributed into a 96-well round bottomed plate. After two washes with PBS-1% FCS, cells were incubated with FcBlock for 15 min at 4°C to reduce non-specific staining. They were then labeled with mAbs or isotype-matched controls for 45 min at 4°C . After incubation, cells were washed twice with PBS-1% FCS, then fixed in 1% formaldehyde and analyzed immediately by flow cytometry.

Cytokine production assays. To determine intracellular cytokine production, 1 ml of each lymph node cell suspension was stimulated with 20 ng/ml phorbol-myristate-acetate (PMA) and 500 ng/ml ionomycin (both from Sigma), and GolgiStop (BD- Pharmingen) for 4.5 h at 37°C under 5% CO_2 . PMA and ionomycin are polyclonal activators known to trigger cytokine release. GolgiStop contains monensin, which inhibits extracellular transport of the cytokines. At the end of incubation, activated cells were washed with cold PBS, resuspended in PBS containing 1% FCS and labeled with anti-CD4-Cy™5 and anti-CD8-FITC at 4°C for 30 min. Cells were then washed in PBS-1% FCS and the pellets were fixed in 100 μl of 4% formaldehyde for 10 min at room temperature in the dark. Fixation was stopped by washing twice in PBS-1% FCS. Cells were permeabilized using saponin-containing Perm/Wash buffer (P/W) according to the manufacturer's recommendations (BD-Pharmingen). They were then distributed into the wells of a 96-well round bottom plate and labeled with anti-IL-2, anti-IL4, anti-IFN- γ mAbs or isotype controls, all conjugated to PE. After incubation for 45 min at room temperature in the dark, cells were washed twice with P/W buffer and finally fixed in 1% formaldehyde before flow cytometry analysis.

Flow cytometry analysis. Simultaneous three-color analysis of cells was performed using a Becton Dickinson FACStar™PLUS flow cytometer run by CELLQuest software. Fluorescence intensities and compensations were calibrated using singularly stained samples. The forward and side scatter properties of lymphocytes were used to gate on viable lymphocytes, excluding erythrocytes, dead cells and debris from the analysis. Surface expression of MHC class II and CD124 molecules were evaluated based on the mean fluorescence intensity (MFI) of single parameter fluorescence histograms. IgE expression was evaluated based on the percentages of IgE $^{+}$ cells in the CD19 $^{+}$ population. Cytokine production was evaluated as the percentage of cytokine-positive cells in the CD4 $^{+}$ and/or CD8 $^{+}$ cell populations. At least 20,000 lymphocytes per sample were doubly gated on physical parameters (forward and side scatter) and positive fluorescence for CD4 or CD8. Data were analyzed using CELLQuest software. The percentages of IgE- and cytokine-positive cells were calculated by statistical analysis of cells contained in quadrants which were set based on the appropriate isotype-matched controls.

Result presentation. To allow the different chemicals tested in separate experiments to be compared, all the parameters, except IL-4, obtained from four individual treated mice were reported as fold change over the respective controls, $\pm$ SD. A fold change value was calculated for each treated mouse using the following formula: Fold change = value obtained from chemical-treated mouse / mean value in the appropriate controls. IL-4 was expressed as mean $\pm$ SD of absolute values from four individual mice, as this cytokine was not produced in control mice.

Statistical analysis. All results are presented as mean $\pm$ SD, which is indicated by error bars in the figures. Statistical analysis was performed using Statgraphics software. The homogeneity of variance was checked using Bartlett's test. When variances were not homogeneous, the data were log-transformed. Data were then subjected to one-way analysis of variance (ANOVA), followed by multiple comparisons analysis (Bonferroni test). Data not meeting the conditions of normality and homogeneity of variance after log transformation were assessed using the Kruskal–Wallis non-parametric test. Logistic regression was also performed with the data obtained in the second set study. P values of 0.05 or less were considered statistically significant. All experiments were repeated at least once with reproducible results. Data shown in this article are from one representative experiment.

Results

First set of chemicals

Chemical concentrations. The chemical concentrations, C, were defined relative to their potential to induce cell proliferation. An LLNA-modified assay was used, in which the number of draining lymph node cells was determined as an indicator of proliferation. Application of 10% TMA, 12.5% PA, 0.5% TDI, 1% DNCB, 0.25% DNFB and 0.25% OXA resulted in a 4.9- to 6.5-fold increase in lymph node cellularity compared to the vehicle control. Likewise, application of one quarter of these doses (i.e., 2.5% TMA, 3.12% PA, 0.12% TDI, 0.25% DNCB, 0.06% DNFB and 0.06% OXA) induced an increase in cell numbers of about 4-fold control values (Table 1). Except the 0.5% TDI dose, which induced a stronger increase in lymph node cell number than 1% DNCB, there was no significant difference between the different chemicals in either range of concentrations. These results indicate that the selected chemical doses have comparable immunogenic potential in terms of inducing sensitization and thus, allow comparison of the immune responses developed in mice following exposure to these doses.

Challenge dose effects. To verify the influence of the challenge dose on the intensity and quality of the local immune response, we tested three challenge concentrations for each chemical. All mice were exposed to a chemical concentration C during sensitization, and to concentrations C, C/2 or C/4 during the challenge phase. No significant difference was found between results for expression of the different markers with the three challenge doses (Figs. 1 and 2). Consequently, the three challenge concentration assays were considered as three independent experiments, and we pooled the results acquired for further statistical analysis.

Analysis of B-cell activation. The B-cell activation state in draining lymph nodes was examined by assessing the membrane expression of maturation markers such as the antigen-presenting MHC class II molecules and co-stimulatory CD86 molecules. To do so, freshly isolated lymph node cells were doubly stained for CD19 and IA ^{d} , or CD19 and CD86. MHC II and CD86 expression levels were assessed on CD19 $^{+}$ cells by measuring the mean fluorescence intensity (MFI) of specific staining. The results, presented as fold change over the respective controls, demonstrated significant differences between the

Table 1

First set of chemicals: selection of allergen concentrations with comparable immunogenic potential using a LLNA-modified assay. BALB/c mice ($n = 4/\text{group}$) were exposed daily on the dorsum of both ears to either vehicle (AOC, acetone/olive oil) (4:1 v/v) or different doses of dissolved chemicals for three consecutive days. Draining auricular lymph nodes were excised 72 h after the last application, pooled for each mouse and counted for cell number determination. Cell number data represent the mean $\pm$ SD for 4 individual mice. Values in italics (*Tr/C*), for chemical-treated groups indicate fold changes in cell number over the respective controls.

Chemical	Category (a)	Concentration	Cell number ($\times 10^6$)	<i>Tr/C</i>	Concentration	Cell number ($\times 10^6$)	<i>Tr/C</i>
AOO	Vehicle		10.6 ± 1.6				
TMA	R-strong	10%	57.0 ± 10.7	5.38	2.5%	44.2 ± 3.6	4.17
PA	R-strong	12.5%	58.6 ± 10.2	5.53	3.12%	47.6 ± 8.9	4.49
TDI	R-strong	0.5%	69.4 ± 2.6	6.55	0.12%	49.6 ± 8.0	4.68
DNCB	C-extreme	1%	58.0 ± 2.5	5.47	0.25%	45.0 ± 4.9	4.25
DNFB	C-extreme	0.25%	68.2 ± 7.8	6.43	0.06%	47.7 ± 13.7	4.50
OXA	C-extreme	0.25%	67.2 ± 8.2	6.34	0.06%	50.3 ± 8.8	4.74

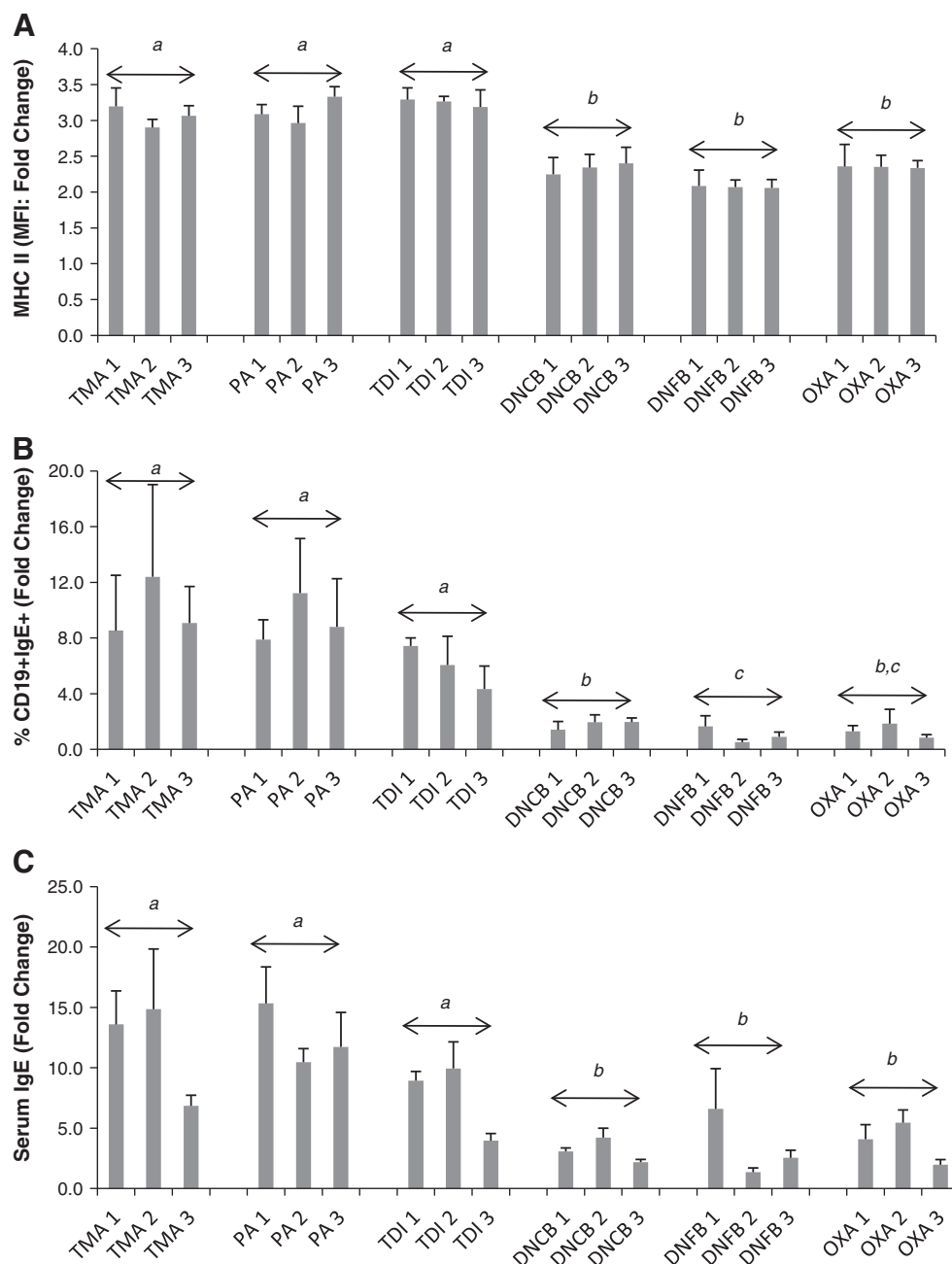


Fig. 1. First set of chemicals: MHC-II and IgE expressions. Flow cytometry analysis of auricular lymph node cells harvested on day 13 from mice sensitized and challenged as described in **Materials and methods**. Mice were sensitized with a single chemical concentration, C, and challenged with three different concentrations, 1 (C), 2 (C/2) and 3 (C/4). Results are expressed as fold change over the respective controls: (A) MHC class II expression by lymph node CD19⁺ B lymphocytes, (B) IgE-positive B lymphocyte percentages, (C) Total serum IgE levels (measured by ELISA in serum samples obtained from the same animals). Bars represent mean $\pm$ SD from 4 individual mice in each group. No significant difference was observed between the 3 challenge concentrations. Therefore, results from these tests were pooled for statistical analysis. Groups of 3 bars with identical letters (^{a b c}) are not statistically different. Difference threshold: $P \leq 0.05$.

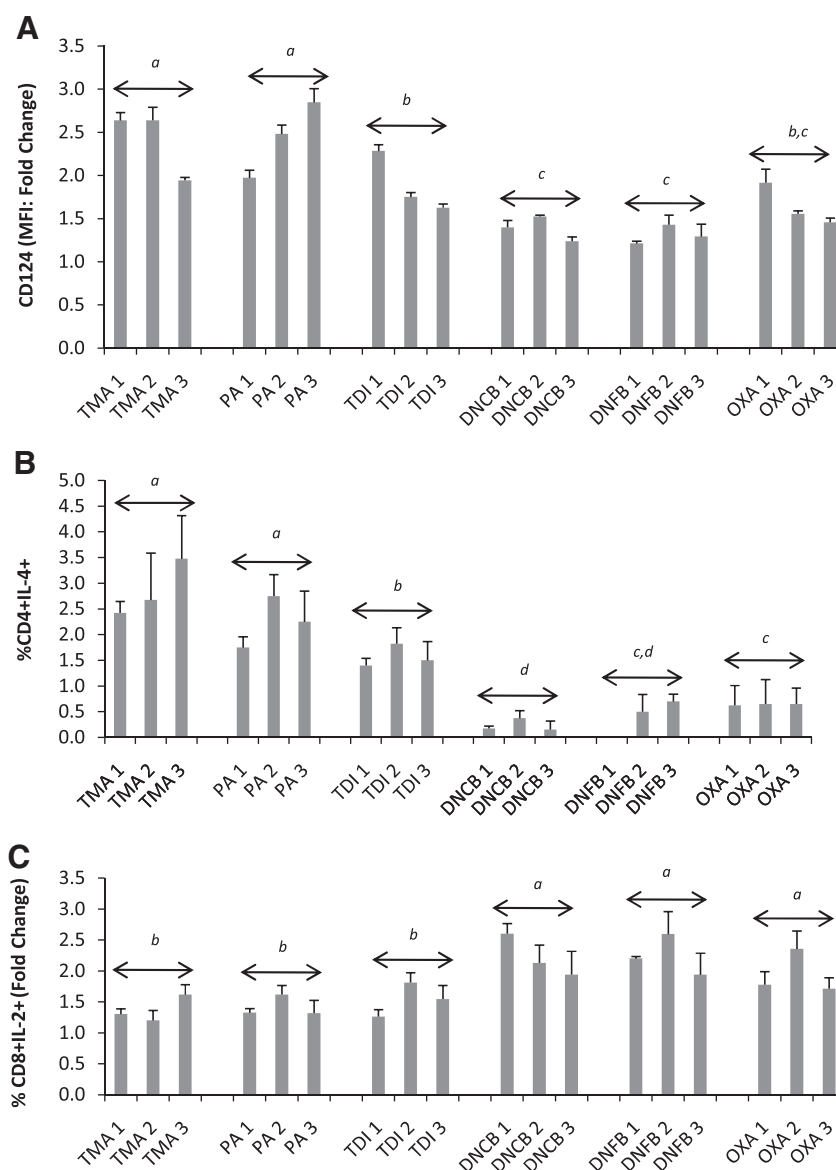


Fig. 2. First set of chemicals: IL-2, IL-4 and CD124 (IL-4 receptor) expressions. Flow cytometry analysis of auricular lymph node cells harvested on day 13 from mice sensitized and challenged as described in [Materials and methods](#). Mice were sensitized with a single chemical concentration, C, and challenged with three different concentrations, 1 (C), 2 (C/2) and 3 (C/4). CD124 and IL-2 results are expressed as fold change over their respective controls. IL-4 results in treated groups are expressed as mean absolute values $\pm$ SD from four individual mice since this cytokine was not produced in control mice. (A) CD124 expression by CD3⁺ T lymphocytes. (B) Intracellular IL-4 production by CD4⁺ T cells. (C) Intracellular IL-2 production by CD8⁺ T cells. Bars represent mean $\pm$ SD calculated from 4 individual mice in each group. Bars with identical letters (^{a b c d}) are not statistically different. Difference threshold: $P \leq 0.05$.

two types of sensitizers ([Fig. 1A](#)). While TMA, PA and TDI consistently induced 3-fold higher levels of MHC-II molecules than their respective controls, contact allergens repeatedly up-regulated MHC-II to levels limited to 2.4-fold changes over controls. Moreover, MHC-II results were very reproducible and the standard deviations, in each group, never exceeded 10%. The trend with expression of the co-stimulatory molecule was for respiratory sensitizers to induce higher levels of CD86 than contact allergens, but these differences were not significant (data not shown).

Analysis of local and systemic IgE production. Local IgE production was measured by assessing the IgE-positive B lymphocytes in the draining lymph nodes. Lymph node cells were doubly stained for CD19 and IgE, and the frequency of IgE⁺CD19⁺ cells was calculated. Typically, exposure to respiratory sensitizers resulted in higher frequencies of IgE⁺CD19⁺ cells than contact allergens ([Fig. 1B](#)). Thus,

the IgE⁺CD19⁺ cell percentages resulted in 4.3- to 12.4-fold changes over controls in TMA-, PA- and TDI-treated mice, while in DNCB-, DNFB- and OXA-treated mice, levels remained unchanged, or never exceeded a 2-fold change. Total serum IgE levels were measured by ELISA in the same animals. Results were in accordance with the percentages of IgE-expressing B cells in local lymph nodes, although the serum IgE concentrations were more variable than the IgE⁺CD19⁺ cell numbers ([Fig. 1C](#)). For both parameters, variance analysis followed by a multiple comparisons test indicated that the results obtained with respiratory sensitizers were significantly different from those obtained with contact sensitizers.

IL-4 receptor alpha (CD124) expression level. Lymph node cells were stained with anti-CD124 and anti-CD3 mAbs. CD124 expression level by CD3⁺ cells was evaluated by measuring the mean fluorescence intensity (MFI) of anti-CD124 staining on the CD3⁺

population. Compared to the respective controls, all sensitizers activated CD124 expression. However, the MFI levels increased differently depending on the sensitizer type (Fig. 2A). TMA and PA induced at least a 2-fold increase in CD124 expression levels, while DNCB and DNFB treatment did not induce more than a 1.5-fold increase. Exposure to TDI and OXA, respectively, increased the CD124 values from 1.6- to 2.3- and 1.5- to 1.9-fold the control values. CD124 levels induced by DNCB, DNFB and OXA were statistically different from those resulting from TMA and PA exposure. Inversely, TMA, PA and TDI induced CD124 levels statistically different from those induced by DNCB.

Intracellular cytokine production: IL-4, IL-2 and IFN- γ . Cytokine production by CD4⁺ and CD8⁺ T cell subpopulations was measured based on the percentages of IL-4, IL-2 and IFN- γ positive cells following stimulation of the lymph node cells for 4.5 h with PMA and ionomycin. Cells were stained for CD4 and CD8, combined with labeling with anti-IL-2, anti-IL-4 or anti-IFN- γ . IL-4 positive cells were almost exclusively CD4⁺. They were either not found in control mice, or were present at very low frequencies not exceeding 0.1% of the CD4⁺ population. Because of this, the frequencies of IL-4⁺CD4⁺ cells observed in treated groups were expressed as absolute values rather than as a fold change over controls, as used for the other cytokines (Fig. 2B). Exposure to respiratory sensitizers, TMA, PA and TDI, resulted in the development of a distinct IL-4 positive population accounting for 1.5% to 3.5% of CD4⁺ cells in the TMA-treated groups. In contrast, contact allergens failed to raise the percentage of IL-4 positive cells beyond 0.7%.

Although IL-2 was produced by both CD4⁺ and CD8⁺ T cells, we were interested in CD8⁺ cells, which demonstrated a greater degree of variation following the different treatments. The percentages of CD8⁺ cells staining positive for IL-2 were expressed as a fold change over their respective controls. DNCB, DNFB and OXA increased the percentages of IL-2 positive CD8⁺ cells to values between 1.8 and 2.6 times the control levels. In comparison, respiratory allergens induced IL-2 production in lower numbers of cells, resulting in fold-change values between 1.2 and 1.8 (Fig. 2C). Despite overlapping was sometimes observed between IL-2 values obtained with TDI and OXA, the statistical analysis of the IL-2 and IL-4 results (ANOVA and multiple comparisons) indicated that the differences between contact and respiratory sensitizers were statistically significant.

IFN- γ production increased significantly following TMA treatment compared to DNCB. However, no difference was observed with exposure to PA, TDI, DNFB and OXA (data not shown).

Second set of chemicals

All the endpoints tested in the first set of chemicals were tested in the second set. However, only results of local IgE production, CD124 expression levels and intracellular IL-4 and IL-2 production are reported. The other endpoints (systemic IgE production, CD86 and MHC class II molecules) were not retained as they were either not or very slightly activated.

Chemical concentrations. To compare the different sensitizers included in this set, doses with comparable immunogenic potential were determined using the LLNA-modified test. Thus, the strong sensitizers included in this group were used at lower doses than those used in the first experiments. The selected doses are presented in Table 2. We considered doses inducing an approximately 3- to 4-fold increase in lymph node cell number to be immunologically similar. The nickel salt was not very soluble in the solvents used for dermal applications, and Eug was toxic when higher doses were applied, therefore, these two compounds were used at doses not inducing this level of stimulation. In this set of compounds, the same concentration of chemical was applied for both sensitization and challenge.

Local IgE production. Exposure to the respiratory sensitizers, TMA, PA, Pt, TDI, TMI and MDI resulted in 3.5- to 10.0-fold increases in IgE⁺CD19⁺ cell percentages over their respective controls (Fig. 3A). Exposure to the contact sensitizers DNCB, Eug and Cr, and the irritant pABA, induced a positive IgE response up to 1.5- to 4.7-fold above control levels. Differences between the two types of sensitizers were not statistically significant. Only the contact allergens HCA and Ni, and the irritant SDS, were significantly different from TMA and other respiratory sensitizers. In the logistic regression, the IgE⁺CD19⁺ cell percentage was not a significant parameter.

Intracellular IL-4 production. With the exception of PA, all the respiratory sensitizers induced IL-4 production in significant numbers of CD4⁺ cells, with IL4⁺CD4⁺ cells percentages between 0.3% and 1.6%. In contrast, the contact sensitizers, DNCB, Eug, HCA Cr and Ni, as well as the irritants SDS and pABA, did not induce IL-4 production or did so at levels not exceeding 0.1% of CD4⁺ cells (Fig. 3B). Statistical analysis indicates significant differences between TMA and all contact sensitizers. However, the levels of IL-4⁺CD4⁺ cells induced by DNCB were not different from those induced by two respiratory sensitizers, PA and MDI. The IL-4 producing CD4⁺ cell percentage was not a significant parameter in the logistic regression.

Table 2

Second set of chemicals: selection of allergen concentrations with comparable immunogenic potential using a LLNA-modified assay. BALB/c mice ($n=4$ /group) were exposed daily on the dorsum of both ears to either vehicle (A00 or dimethyl sulfoxide DMSO) or different doses of dissolved chemicals for three consecutive days. Draining auricular lymph nodes were excised 72 h after the last application, pooled for each mouse and counted for cell number determination. Cell number data represent the mean $\pm$ SD for 4 individual mice. Values in italics (*Tr/C*), for chemical-treated groups indicate fold changes in cell number over the respective controls.

Chemical/concentration	Category	Cell number ($\times 10^6$)	<i>Tr/C</i>	Chemical/concentration	Category	Cell number ($\times 10^6$)	<i>Tr/C</i>
TMA 2.5%	R-strong	44.2 $\pm$ 8.9	4.16	DNCB 0.25%	C-extreme	45.0 $\pm$ 4.9	4.24
A00		10.6 $\pm$ 1.6		A00		10.6 $\pm$ 1.6	
PA 3.12%	R-strong	47.6 $\pm$ 8.9	4.49	Eug 30%	C-weak	12.9 $\pm$ 2.6	2.68
A00		10.6 $\pm$ 1.6		A00		4.8 $\pm$ 0.5	
Pt 0.25% (x2)	R-strong	29.3 $\pm$ 5.7	3.02	HCA 30%	C-moderate	23.1 $\pm$ 2.3	4.81
DMSO		9.7 $\pm$ 1.3		A00		4.8 $\pm$ 0.5	
TDI 0.25%	R-strong	38.2 $\pm$ 4.1	3.89	Cr 0.25% (x2)	C-strong	30.9 $\pm$ 3	3.18
A00		9.8 $\pm$ 0.5		DMSO		9.7 $\pm$ 1.3	
TMI 0.25%	R-?	39.2 $\pm$ 8.0	4.78	Ni 5% (x2) DMSO	C-moderate	4.7 $\pm$ 2.5	2.53
A00		8.2 $\pm$ 1.1				9.7 $\pm$ 1.3	
MDI 0.25%	R-strong	37.1 $\pm$ 3.7	4.52	SDS 20% (x2) DMSO	Irritant	21.4 $\pm$ 2.4	2.20
A00		8.2 $\pm$ 1.1				9.7 $\pm$ 1.3	
				PABA 10% A00	Irritant	6.2 $\pm$ 1.2	1.19
						5.2 $\pm$ 0.3	

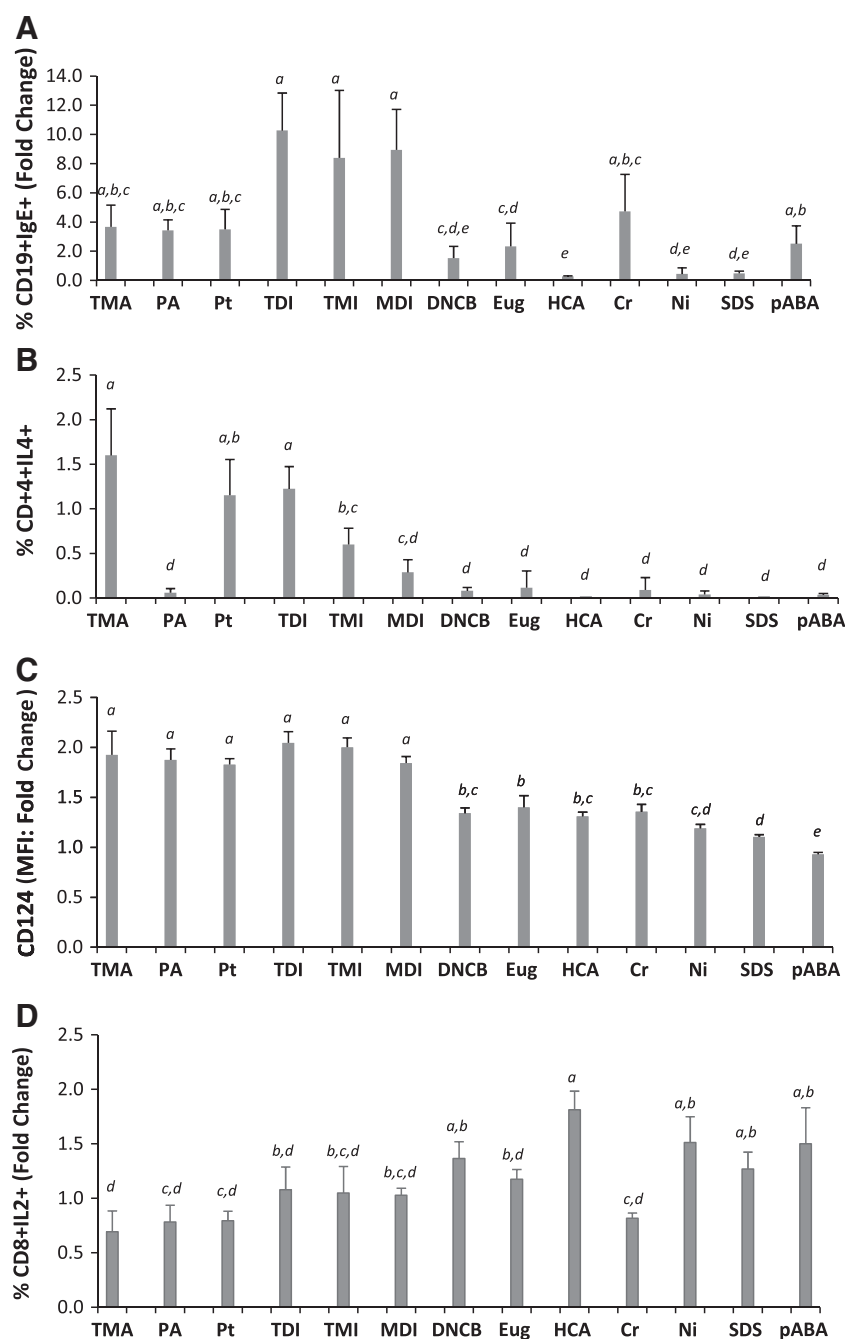


Fig. 3. Second set of chemicals. Flow cytometry analysis of auricular lymph node cells harvested on day 13 from mice sensitized and challenged as described in [Materials and methods](#). A unique concentration of each chemical was used throughout the treatment. IL-4 is expressed as mean absolute values $\pm$ SD from four individual mice since this cytokine was not produced in control mice. All other results are expressed as fold change over their respective controls. (A) IgE-positive B lymphocyte percentages. (B) Intracellular IL-4 production by CD4⁺ T cells. (C) CD124 (IL-4 receptor) expression by CD3⁺ T lymphocytes. (D) Intracellular IL-2 production by CD8⁺ T cells. Bars represent mean $\pm$ SD calculated from 4 individual mice in each group. Bars with identical letters (*a b c d e*) are not statistically different. Difference threshold: $P \leq 0.05$.

IL-4 receptor- α (CD124) expression level. All sensitizers up-regulated membrane expression of CD124 on CD3⁺ T cells, but different induction levels were observed depending on the sensitizer type. Exposure to the six respiratory sensitizers produced increases in CD124 expression between 1.8- and 2.0-fold control values, while contact allergens induced lower levels of CD124, resulting in 1.2- to 1.4-fold changes (Fig. 3C). Exposures to the chemical irritants SDS and pABA were not associated with any change in CD124 expression, with fold change values of 1.1 and 0.9, respectively. ANOVA and multiple comparisons tests demonstrated a significant difference in CD124 expression between the two groups of allergens. Logistic regression, classed CD124 as a good discriminant parameter with a 100% predictive capacity.

Intracellular IL-2 production. With the exception of the chromium salt, all contact sensitizers and irritants increased the percentage of IL-2-producing CD8⁺ lymphocytes to between 1.2- and 1.8-fold control levels. Conversely, the IL-2⁺CD8⁺ cell percentages were reduced following Cr exposure, with a 0.8-fold change (Fig. 3D). The respiratory sensitizers, TMA, PA and Pt reduced IL-2⁺CD8⁺ cell frequencies to 0.7- to 0.8-fold control values. The three isocyanates, TDI, TMI and MDI, in contrast had no, or a very slight, effect on this parameter, with fold changes close to 1. ANOVA and multiple comparisons tests showed that TMA, PA and Pt were significantly different from the contact allergens DNCB, HCA and Ni and from the irritants. The three isocyanates, Eug and Cr were not really distinguished. However,

the logistic regression classed IL-2 as a discriminant parameter with a predictive capacity of 81.8%.

Plot representation of IL-2 and CD124 values. The plot representation of numbers of IL-2 positive CD8⁺ cells against CD124 expression by CD3⁺ cells separates the different chemicals into two distinct populations, as illustrated in Fig. 4. All the respiratory sensitizers are positioned in an area of high CD124 and low IL-2 expression, while contact sensitizers and irritants are found in a region of lower CD124 and higher IL-2 expression.

Discussion

The data presented in the first part of this study support the findings of a previous study (Goutet et al., 2005) which reported that the local immune responses induced in mice cutaneously exposed to the respiratory and contact chemical allergens, TMA or DNCB, could be distinguished by changes in IgE production, MHC class II and CD124 expression and intracellular IL-2, IL-4 and IFN- γ production. Indeed, we ensured that other strong sensitizers, such as the respiratory sensitizers PA and TDI and the contact sensitizers DNFB and OXA, induced similar immune responses to those respectively produced by TMA and DNCB. Using the previously described protocol, mice were exposed to allergen doses demonstrating comparable immunologic potential in a LLNA-modified assay.

In comparison to contact sensitizers, respiratory sensitizers TMA, PA and TDI increased the percentages of IgE-positive B cells in lymph nodes and the total serum IgE levels, confirming our earlier results, and those of Manetz and Meade (1999). However, serum IgE values were more variable than the IgE-positive B-cell percentages, making cytometry-based IgE⁺CD19⁺ cell analysis more reliable to characterize chemical sensitizers than total serum IgE.

Similarly, the expression level of MHC class II molecules on B cells was linked to the sensitizer type. Throughout the experiments, respiratory sensitizers induced higher density of MHC class II than contact allergens. Increased MHC-II expression is thought to activate B lymphocytes in potent antigen-presenting cells and potent stimulators of CD4⁺ and CD8⁺ T cells (Murray, 1998). The differences in MHC-II expression observed between the allergen types may account for the specific induction of Th1 or Th2 type immune responses (Schountz et al., 1996). Thus, this parameter might be suitable to assess the ability of highly immunogenic allergens to induce either respiratory or contact allergy.

Finally, divergent profiles were observed in terms of intracellular IL-2 and IL-4 production. In comparison with the contact sensitizers, TMA, PA and TDI induced IL-4 synthesis in larger numbers of CD4⁺ cells as well as an over expression of the IL-4 R α . In contrast, DNCB,

DNFB and OXA, induced IL-2 synthesis in greater percentages of CD8⁺ cells. Thus, except for IFN- γ , which we found here to increase similarly with all sensitizers, the current results confirm that IgE-positive B cells, MHC class II, IL-4R α , IL-4 and IL-2 might be efficient markers to discriminate between strong respiratory and contact allergens.

The second, and most important, part of our study focused on chemicals with lower sensitizing potential. Our aim was to ensure that markers capable of identifying high doses of strong allergens could also effectively identify lower doses, or weakly immunogenic chemicals which are most often encountered in the industrial sector. Six respiratory allergens, including TMA, PA, Pt, TDI, TMI and MDI, and five contact allergens, including DNCB, Eug, HCA, Cr and Ni, were compared. We also tested pABA and SDS, which are irritants in human pathology. SDS has been widely used as a negative control for the validation of a number of animal sensitizing experiments, although it induces false-positive results in LLNA (Basketter et al., 1998). While this panel includes different categories of chemicals, all compounds were tested at doses of equivalent immunogenicity in line with the concentrations of the reference molecules, 2.5% TMA and 0.25% DNCB (instead of 10% and 1% in the first part of the study). Results indicated that CD124 and, to a lesser extent, IL-2 have the potential to distinguish moderate immune responses induced by respiratory and contact sensitizers. In contrast, MHC-II (data not shown), IgE and IL-4 were not decisive indicators.

Analysis of IgE-positive B cells in the second panel gave divergent results. Three out of six respiratory allergens, TMA, PA and Pt, induced levels of IgE⁺ B cells similar to those induced by the contact sensitizers DNCB, Eug, and Cr, and the irritant pABA. In addition, the IgE⁺ B-cell percentages induced by TMA and PA were 2-fold lower than those observed in the first panel. These differences may result from effects on IgE production kinetics, caused by the chemical properties of the sensitizers and the reduced doses applied, as reported in previous studies (Guo et al., 2002; Zhang et al., 2002). To verify this hypothesis, we checked how the IgE⁺ B-cell population evolved in TMA- and Pt-exposed mice over a longer period of time: 18 rather than 13 days. In these conditions, the IgE⁺ B-cell numbers increased, indicating that IgE synthesis was truly delayed (data not shown). Because of these variations in IgE production kinetics, this marker cannot be reliably used to classify low or moderate allergens.

Like IgE production, IL-4 synthesis observed with the second panel did not confirm the results obtained with higher allergen doses. Only four of the six respiratory allergens induced significantly higher IL-4 levels. PA and MDI resulted in low IL-4 levels, similar to those induced by contact allergens and irritants. IL-4 has often been identified as a protein of choice for sensitizer classification in previous studies but the methods used generally focused on strong allergic reactions (Dearman et al., 2002; Plitnick et al., 2002). Indeed, other studies reported that IL-4 production was dependent on the allergen doses applied (De Jong et al., 2009; Van Och et al., 2002). Moreover, IL-4 might not be detected as it is known to be a short-lived protein produced over a short period of time (Kroegel et al., 1996). Thus, the inherent nature of IL-4 limits its usefulness to identify weak allergens.

Despite this, IL-4 is believed to play a major role in the development of atopic asthma by directing the immune system towards a Th2 response. In association with IL-13, a closely related cytokine, IL-4 controls Th2 cell proliferation, IgE-expressing B-cells promotion, eosinophil differentiation and activation and the stimulation of mucus producing cells (Balic et al., 2006; Ubo et al., 1999). IL-4 and IL-13 exert their biological activities through binding to a common subunit of their respective receptor complexes, the IL-4R α chain, or CD124, mainly expressed on the surface of T cells. These bonds initiate an interactive process, critically regulating the immune response (Andrews et al., 2006). Previous studies reported that mice deficient in the IL-4R α chain did not produce IgE (Noben-Trauth et al., 1997), indicating a crucial role for signaling via this receptor in production of this

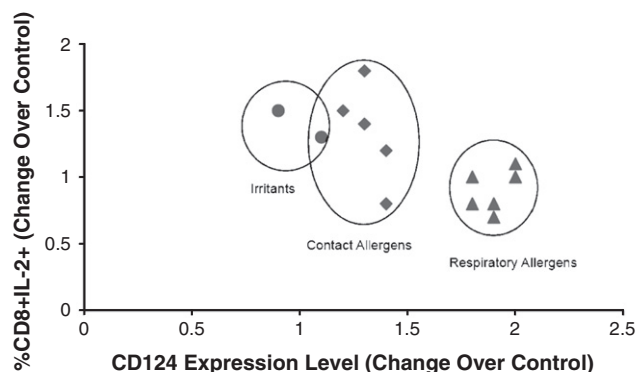


Fig. 4. Plot representation of IL-2 positive CD8⁺ cells against IL-4 receptor (CD124) expression in CD3⁺ cells, both expressed as fold change over their respective controls. Each point represents the mean $\pm$ SD for 4 individual mice (respiratory sensitizers ▲, contact sensitizers ■ and irritants ⊗).

protein. Analyses of human bronchial biopsy specimens demonstrated that IL-4R α is constitutively expressed in normal airways, and that expression is enhanced in tissue from asthmatic individuals (Kotsimbos et al., 1998). Throughout this study, the CD124 expression levels were linked to the sensitizer type. Discriminative CD124 expression was observed with the first group of chemicals, where the three respiratory allergens induced significantly higher expression levels than the reference contact allergen, DNCB. In contrast, the three contact allergens generated lower levels of CD124 than the reference respiratory allergen, TMA. However, the respiratory allergen TDI and the contact allergen OXA induced similar CD124 levels, demonstrating that allergens are best identified by comparison with reference sensitizers such as TMA and DNCB (Toebak et al., 2006). Indeed, this apparent discrepancy observed between TDI and OXA may result, once again, from the specific properties of these chemicals, generally known to induce mixed immune responses modulating the expression of both Th1 and Th2 parameters (De Jong et al., 2009; Farraj et al., 2007; Selgrade et al., 2006; Ulrich et al., 2001). In the second study, the six respiratory allergens induced repeatedly higher CD124 levels than the contact allergens, while irritants had no effect. Compared with IgE and IL-4, which often displayed important inter-individual variability, CD124 results are very uniform. Throughout this study, the mean standard deviations in each group were less than 5%.

The relevance of CD124 when identifying allergens had been recently observed in a study designed to compare the pulmonary immune responses induced in mice intratracheally exposed to chromium or platinum salts (Ban et al., 2010). In this study, mice exposed to Pt developed a Th2 immune response, linked to an increase in CD124 expression from the beginning of treatment. In contrast, Cr-exposed mice showed no increase in CD124 expression, but developed, first, a mixed immune response which turned into a Th1 response three weeks later. This suggests a role for CD124 in orienting the immune response. Likewise, CD124 expression following TMI exposure in the second set study may be significant. While TDI and MDI are well known respiratory allergens, more information is needed about TMI to class it. Plitnick et al. (2005) provisionally classified TMI as a weak respiratory allergen because of its ability to induce a Th2 cytokine profile. Farraj et al. (2007) reported that intranasal exposure of mice to TMI induced similar pulmonary responses to TDI and MDI. Our results provide additional elements, showing that TMI amplifies CD124 expression like other respiratory sensitizers. Taken together, these observations confirm that CD124 can directly help drive a Th2 response, thus making it a relevant marker for allergen classification.

While CD124 plays a pivotal role in orienting the Th2 response in allergic asthma, IL-2 polarizes the development of a Th1 response in contact dermatitis (Mosmann and Sad, 1996). The IL-2 action is essential to stimulate proliferation and activation of Th1 lymphocytes, and to enhance cytotoxicity. IL-2 is naturally produced in mice by CD4⁺ and CD8⁺ T cells. In this study, exposure to contact or respiratory sensitizers induced differential effects on IL-2 production, mainly in the CD8⁺ population. The IL-2⁺CD8⁺ cell values were discriminative in the first set study, but it classed compounds in the second set less effectively, as Cr, Eug and the three isocyanates were not clearly distinguished. Nonetheless, the contact allergens and irritants showed a significant tendency to induce IL-2 at higher levels than respiratory allergens. The IL-2 production induced by SDS and pABA may result from non-specific processes, part of the inflammatory reactions provoked by these compounds as previously reported (Cumberbatch et al., 2002). We also examined the discriminating potential of CD25, the IL2-R α chain, part of the high affinity IL-2 receptor complex (Smith, 1988). CD25 expression tended to mimic IL-2 expression, but the results were not significant (data not shown). To date, IL-2 production has not been widely investigated as marker to identify allergenic chemicals. IL-2 mRNA expression could not distinguish allergen subsets (Manetz et al., 2001; Plitnick et al., 2002).

Our data relating to intracellular IL-2 production are coherent with the role of IL-2 in contact allergies and demonstrate that this marker might be usefully associated with CD124 to classify chemical allergens. Also, the inverse relationship between IL-2 production and CD124 expression might reinforce the classification.

In summary, we first demonstrated that highly immunogenic respiratory and contact chemical allergens may be differentiated by several immune markers included IgE-positive B cells, MHC II and CD124 expression, IL-4-producing CD4⁺ cells and IL-2-producing CD8⁺ cells. Secondly, we verified that only two of these markers, CD124 and IL-2, have the potential to classify chemical allergens with moderate or low immunogenic potential. Neither IgE nor IL-4 production was distinctive, although they may provide additional information. While this approach has been tested on a limited number of sensitizers, it may lead to improved characterization of chemical allergens by allowing a variety of sensitizers to be screened.

Conflict of interest statement

None.

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