

# Skin Exposure to Weak and Moderate Contact Allergens Induces IFN $\gamma$ Production by Lymph Node Cells of CD4 + T-Cell-Depleted Mice

Marc Vocanson<sup>1,2,3</sup>, Anca Hennino<sup>2,3</sup>, Aurore Rozières<sup>1,2,3</sup>, Magalie Cluzel-Tailhardat<sup>2</sup>, Gaelle Poyet<sup>1,2,3</sup>, Magalie Valeyrie<sup>2</sup>, Josette Bénétière<sup>1,2,3</sup>, Rosine Tédone<sup>2</sup>, Dominique Kaiserlian<sup>2,3</sup> and Jean-François Nicolas<sup>1,2,3,4</sup>

Allergic contact dermatitis (ACD) is mediated by hapten-specific CD8 + T cells and downregulated by CD4 + T cells. We have recently shown in a model of ACD to weak haptens that priming of IFN $\gamma$ -producing CD8 + T cells and the development of skin inflammation could be obtained in mice deficient in CD4 + T cells. Here we show that IFN $\gamma$  production by lymph node (LN) cells draining the site of skin sensitization of CD4 + T-cell-deficient mice is a marker of the sensitizing properties of weak haptens. LN cells from mice sensitized as in the classical local lymph node assay (LLNA) were recovered at day 5, then cultured for 20 hours in the presence of submitogenic doses of phytohemagglutinin, and finally tested for the production of IFN $\gamma$ . Results show that: (i) production of IFN $\gamma$  by LN cells was induced by weak and moderate allergens in a dose-dependent fashion; (ii) the magnitude of IFN $\gamma$  production paralleled the sensitizing properties of allergens allowing to classify them as moderate or weak haptens; (iii) chemicals without sensitizing properties were unable to stimulate IFN $\gamma$  production by LN cells. Therefore, the IFN $\gamma$  LLNA appears as a sensitive, specific, and robust assay to detect weak contact allergens.

*Journal of Investigative Dermatology* advance online publication, 13 November 2008; doi:10.1038/jid.2008.352

## INTRODUCTION

Allergic contact dermatitis (ACD) is major occupational and environmental health issue in most industrial countries (Blauvelt *et al.*, 2003; Saint-Mezard *et al.*, 2004b; Coenraads and Goncalo, 2007). ACD, also referred to as contact hypersensitivity (CHS), is a T-cell-mediated skin inflammatory reaction following repeated contact of individuals with non-protein sensitizing chemicals, called haptens, that is, contact allergens or contact sensitizers. Depending on the ability with which haptens induce sensitization on exposed individuals, they are categorized as strong, moderate, or weak sensitizers (Gerberick *et al.*, 2004). Although strong haptens easily sensitize naïve individuals at very low doses, weak allergens, which comprise most of the chemicals involved in human ACD, can only induce sensitization in a small proportion of individuals and at much higher doses.

The pathophysiology of CHS classically involves two phases, that is, the sensitization and elicitation phases (Saint-Mezard *et al.*, 2004b). The sensitization phase (afferent phase) occurs after skin contact with a hapten leading to the priming of hapten-specific IFN $\gamma$ -producing CD8 + T cells in the lymph nodes (LNs) draining the site of exposition (Saint-Mezard *et al.*, 2004a). The elicitation phase occurs when the sensitized individual is re-exposed to the same allergen. CD8 + T cells are then recruited and activated at the site of skin contact, where they initiate the inflammatory process leading to the cutaneous lesions through IFN $\gamma$  production and cytotoxicity toward keratinocytes (Trautmann *et al.*, 2001; Akiba *et al.*, 2002). Hence, today it is widely accepted that IFN $\gamma$  producing CD8 + T cells are the dominant effector population of CHS which can develop in the absence of CD4 + T-cell help (Martin, 2004; Saint-Mezard *et al.*, 2004a; Vocanson *et al.*, 2006b). Moreover, accumulating evidence has shown that CD4 + T cells (notably the CD4 + CD25 + FoxP3 + T-cell subset (Sakaguchi *et al.*, 2006)) limit the priming of CD8 + effector T cells in the draining LNs and participate to the resolution of the skin inflammatory reaction (Dubois *et al.*, 2003). We recently demonstrated that CD8 + T cells are effector cells of CHS to strong haptens as well as to common weak haptens. Whereas CHS responses to strong haptens, for example DNFB, are obtained in normal mice, CHS reactions in response to sensitization to weak haptens occurred only in CD4 + T-cell-deficient mice, that is, mice deficient in regulatory CD4 + T

<sup>1</sup>Faculté de Médecine, Université Lyon1, Lyon-Sud, France; <sup>2</sup>INSERM, U851, Lyon, France; <sup>3</sup>Université Lyon, IFR 128, Lyon, France and <sup>4</sup>Hospices Civils de Lyon, Immunologie Clinique et Allergologie, CH Lyon-Sud, France

Correspondence: Dr Marc Vocanson, INSERM U851, IFR 128, BioSciences Lyon-Gerland, 21 Avenue Tony Garnier, 69365 Lyon Cedex 07, France. E-mail: marc.vocanson@inserm.fr

Abbreviations: ACD, allergic contact dermatitis; CHS, contact hypersensitivity; LLNA, local lymph node assay; LN, lymph node; mAb, monoclonal antibody; PHA, phytohemagglutinin; SFC, spot-forming cell; SI, stimulation indices; WT, wild type

Received 27 May 2008; revised 1 September 2008; accepted 9 September 2008

cells (Vocanson *et al.*, 2006b). In this system, CHS to weak haptens displayed all the features of a classical CHS to strong haptens and was mediated by IFN $\gamma$  secreting CD8 + T cells primed in skin-draining LNs, the days following sensitization.

For many years, hazard identification of skin sensitizers relied on animal models such as the guinea pig maximization test, the occluded patch test of Buehler, and the murine ear-swelling test (Maurer, 2007). Chemicals able to induce a CHS response are identified as skin sensitizers in those tests. More recently, the murine local lymph node assay (LLNA) (Kimber and Dearman, 1991; Kimber *et al.*, 2002) was designed to appreciate the events occurring during the sensitization phase in naïve animals. This assay measures the proliferative response of draining LN cells provoked by repeated topical application of the test chemical. Hence, the LLNA has brought tangible benefits for immunotoxicological purposes: (i) accurate detection of common contact sensitizers that can be classified according to their sensitization properties (strong, moderate, weak) (Gerberick *et al.*, 2004); (ii) reduction of the number of animals used (50%) and improvement in animal welfare (Kimber *et al.*, 2002). However, the LLNA features some negative aspects among which the use of radioactivity and the absence of specificity, leading to false positive responses with some chemicals devoid of sensitizing properties but endowed with irritant pro-inflammatory properties. Moreover, the detection of chemicals endowed with weak sensitizing activity turned out to be difficult. Therefore, there is still considerable interest in improving the alternative methods for detection of the sensitizing properties of chemicals.

As CHS to common contact allergens is mediated by hapten-specific IFN $\gamma$ -producing CD8 + T cells and because IFN $\gamma$  is a cytokine whose production is restricted to activated T cells, we postulated that production of IFN $\gamma$  by LN T cells draining the sensitization site could reflect hapten-specific effector T-cell priming and therefore could be used to characterize the sensitizing properties of chemicals. We and others have previously shown that strong haptens like DNFB, DNCB, or oxazolone potently activate IFN $\gamma$  production in LNs, draining the sensitization site in mice using LLNA-derived protocols (Kehren *et al.*, 1999; Akiba *et al.*, 2002; Saint-Mezard *et al.*, 2004a; Azam *et al.*, 2005). In contrast, when weak/moderate haptens were tested, the magnitude of the IFN $\gamma$  response was routinely low and the results were poorly reproducible (Azam *et al.*, 2005; Vocanson *et al.*, 2006b). In the present study, we show that depletion of CD4 + T cells in weak hapten-sensitized mice greatly improves the IFN $\gamma$  production by LN cells, in an LLNA-modified protocol. From this observation, we propose a new alternative method, referred to as the IFN $\gamma$  LLNA, to detect common contact allergens.

## RESULTS

To study the skin allergenic activity of different chemicals, wild type (WT) and CD4 + T-cell-depleted BALB/c mice were sensitized by repeated skin painting (days 0, 1, and 2) with chemicals on both ears, according to the standard LLNA

protocol (Kimber and Dearman, 1991; OECD and Chemicals GfT, April 2002). *In vivo* priming of hapten-induced T cells was assessed by IFN $\gamma$ -ELISA and -ELISPOT assays performed on LN cells recovered from immunized mice, and restimulated *in vitro* with submitogenic doses of phytohemagglutinin (PHA). This test was named IFN $\gamma$  LLNA.

### CD4 + T-cell depletion leads to increased IFN $\gamma$ production by LN cells of weak hapten-sensitized BALB/c mice

We previously reported that ACD to weak/moderate haptens is mediated by IFN $\gamma$ -producing CD8 + T cells under the control of CD4 + T cells endowed with suppressive/regulatory functions (Vocanson *et al.*, 2006b). Figure 1 shows CD4 + T-cell depletion of sensitized WT BALB/c mice results in increased IFN $\gamma$  production by LN cells compared with undepleted mice. Detectable but very low IFN $\gamma$  (0.016–0.050 ng ml<sup>-1</sup>) was produced at day 5 by LN cells of undepleted WT mice sensitized with two common fragrance allergens (hexylcinnamaldehyde (HCA) and hydroxycitronellal (HDCL)). In contrast, LN cells from mice acutely depleted in CD4 + T cells produced high quantities of IFN $\gamma$  (3.595–13.8 ng ml<sup>-1</sup>). Stimulation indices (SI) obtained by comparison of IFN $\gamma$  production by LN cells from chemical-sensitized versus vehicle-sensitized mice were respectively 6 and 2 for HCA and HDCL in undepleted mice, and 23 and 7 in CD4 + T-cell-depleted mice. Hence, the detection of the sensitizing activity of weak sensitizers is dramatically increased in CD4 + T-cell-depleted mice compared with normal animals.

### Development of the IFN $\gamma$ LLNA

On the basis of the above results, development of the IFN $\gamma$  LLNA was done using CD4 + T-cell-depleted BALB/c mice only and HCA which is a recommended LLNA positive control (Dearman *et al.*, 2001). Figure 2 shows the dose-response effect of PHA on IFN $\gamma$  production by LN cells of HCA- and vehicle-sensitized mice, in a 20 hours culture system. IFN $\gamma$  secretion of LN cells from vehicle (acetone/olive oil (A/OO, 4:1))-painted mice increased mildly with PHA concentration and reached a plateau at high mitogenic doses (Figure 2a and b). In contrast, the production of IFN $\gamma$  by LN cells from HCA-sensitized mice increased dramatically with PHA concentration, reached a plateau at 20  $\mu$ g ml<sup>-1</sup> and dropped at 40  $\mu$ g ml<sup>-1</sup> (Figure 2a and b). Time-course experiments indicated that IFN $\gamma$  secretion rose progressively in the first hours of PHA stimulation was maximal at about 20 hours and decreased in the following hours (Figure 2c).

As expected, SI inversely correlated with the concentration of PHA (Figure 2b). Indeed, at high doses, PHA exerts mitogenic activity toward the entire LN T-cell subset leading to non-specific IFN $\gamma$  production. In contrast, at low (sub-mitogenic) doses below 10  $\mu$ g ml<sup>-1</sup>, PHA was able to induce IFN $\gamma$  production by LN cells that had been activated *in vivo* only, suggesting that the response obtained with HCA reflects the activation of *in vivo* hapten-induced T cells.

Finally, the kinetics of the HCA-induced T-cell response was monitored at different days after sensitization by IFN $\gamma$ -ELISPOT. IFN $\gamma$  spot-forming cells (SFCs) were detected as

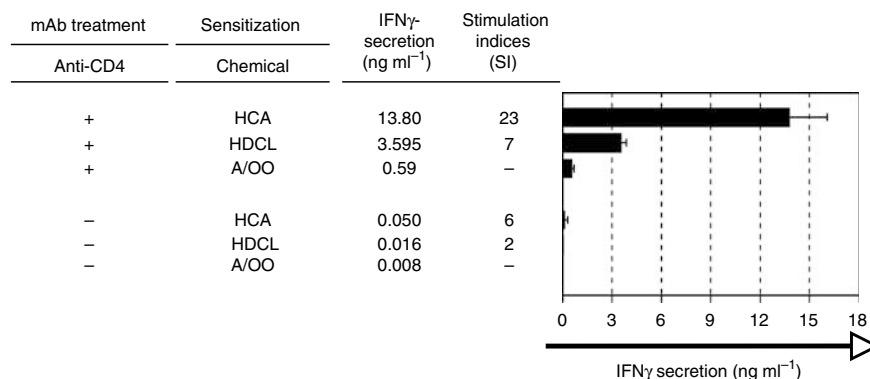


**Figure 1. IFN $\gamma$  production by LN cells is dramatically increased in CD4 + T-cell-depleted mice compared with normal mice on hapten sensitization.** Normal or CD4 + T-cell-depleted BALB/c mice were sensitized on both ears for three consecutive days (days 0, 1, 2) by applications of 25% (w/v) HCA, 25% (w/v) hydroxycitronellal (HDCL), or the vehicle A/OO. At day 5, draining LN cells were collected and cultured *ex vivo* in presence of 5  $\mu$ g ml $^{-1}$  PHA for 20 hours. IFN $\gamma$  secretion was titrated by ELISA. Results are represented as raw data and histograms. Stimulation indices obtained by comparison of IFN $\gamma$  production by LN cells from chemical-sensitized versus vehicle-sensitized mice (SI = IFN $\gamma$  secretion for HCA or HDCL-sensitized mice/IFN $\gamma$  secretion for AOO sensitized-mice) are reported. Results are representative of three different experiments with 4 mice per group.

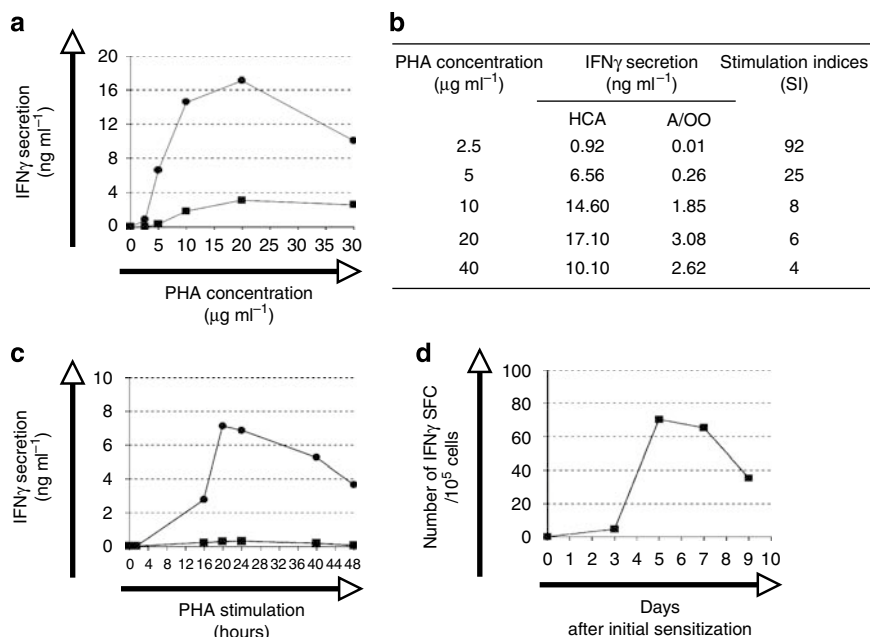


**Figure 2. Development of the IFN $\gamma$  LLNA in CD4 + T-cell-depleted mice.** (a, b) Influence of the PHA concentration on IFN $\gamma$  secretion. CD4 + T-cell-depleted BALB/c mice were sensitized on both ears for three consecutive days (days 0, 1, and 2) by applications of 25% (w/v) HCA (●) or the vehicle A/OO (■). At day 5, draining LN cells were collected and cultured *ex vivo* for 20 hours in presence of different concentrations of PHA. IFN $\gamma$  secretion was measured by ELISA assay. Results are represented as graph (a) and raw data (b). Stimulation indices obtained by comparison of IFN $\gamma$  production by LN cells from HCA-sensitized versus vehicle-sensitized mice (SI = IFN $\gamma$  secretion for HCA-sensitized mice/IFN $\gamma$  secretion for AOO sensitized-mice) are reported (b). Results are representative of three different experiments with four mice per group. (c) Influence of the time of PHA stimulation on IFN $\gamma$  secretion. CD4 + T-cell-depleted BALB/c mice were sensitized as described above by applications of 25% (w/v) HCA (●) or the vehicle A/OO (■). At day 5, draining LN cells were collected and cultured *ex vivo* in presence of 5  $\mu$ g ml $^{-1}$  PHA for different intervals of time. IFN $\gamma$  secretion was titrated by ELISA assay. Results are representative of three different experiments with four mice per group. (d) Kinetics of the HCA-induced T-cell response. CD4 + T-cell-depleted BALB/c mice were sensitized on both ears for three consecutive days (days 0, 1, and 2) by applications of 25% (w/v) HCA. At different intervals of time after the first sensitization, draining LN cells were collected and cultured *ex vivo* with 5  $\mu$ g ml $^{-1}$  of PHA. IFN $\gamma$  spot-forming cells (SFCs) were determined after 20 hours of cell culture. Results are representative of three different experiments with four mice per group.

soon as day 3, peaked at day 5, and decreased in the following days (Figure 2d).

Of note, similar results, including dose and time-course responses, were obtained with another mitogenic agent for T

cells, that is, anti-CD3 monoclonal antibody (mAb) (2c11 clone) used at submitogenic concentrations (Figure S2).

From the above data, the following parameters were chosen for the test: (i) LN cells are recovered at day 5 post-

sensitization; (ii)  $5 \mu\text{g ml}^{-1}$  is the dose of PHA inducing IFN $\gamma$  production by sensitized mice but not vehicle-treated mice; (iii) as ELISA and ELISPOT gave similar results (Figure S3), ELISA was chosen for the detection of IFN $\gamma$  production by LN cells because it is easier to perform than ELISPOT.

#### Inter-experiment standardization of the IFN $\gamma$ LLNA results

During the development of the test, we observed that the magnitude of IFN $\gamma$  secretion by LN cells of vehicle- and HCA-painted mice varied greatly, but in similar proportions, from one experiment to another. Therefore, in order to compare results obtained from different experiments, HCA was introduced in each experiment and the results recorded for test haptens were expressed as a percentage of the HCA response. All chemicals were tested at a concentration of 25% (w/v) except sodium lauryl sulfate (SLS), which was used at 10% (w/v) (it was not soluble at higher doses in the two vehicles—A/OO or *N,N*-dimethylformamide (DMF)—used in this study). Respective molar concentrations were: isoeugenol (1.52 M), HCA (1.16 M), dihydrocoumarin (DHC) (1.59 M), lylal (1.19 M), methyl octine carbonate (MOC) (1.49 M), eugenol (1.52 M), HDCL (1.45 M), lilial (1.22 M), geraniol (1.62 M), coumarin (1.71 M), SLS (0.35 M), isopropalol (ISOP) (4.16 M), and methyl salicylate (MS) (1.64 M).

#### The IFN $\gamma$ LLNA detects the sensitizing properties of weak haptens

Several groups of chemicals were tested in the IFN $\gamma$  LLNA: (i) non-sensitizers (ISOP, MS, SLS); (ii) weak haptens (eugenol, geraniol, HDCL, lilial, lylal, MOC, coumarin), and (iii) moderate haptens (isoeugenol, HCA, DHC).

The IFN $\gamma$  LLNA responses, that is, IFN $\gamma$  production by PHA-restimulated LN cells, induced by ear painting of different moderate/weak haptens, were dose-dependent (Figure 3). Conversely, responses induced by increasing doses of the non-sensitizer MS only slightly exceeded the response induced by the vehicle A/OO.

Interestingly, the IFN $\gamma$  LLNA responses induced by non-sensitizers (ISOP, MS, SLS) and by the vehicles (A/OO and DMF) always represented less than 15 and 5% of that induced by HCA, respectively. Therefore, a threshold value of 15% of the HCA-induced IFN $\gamma$  LLNA response was

chosen to identify chemicals endowed with sensitizing properties.

In this context, all the weak sensitizers tested gave positive responses in the test (Figure 4). Isoeugenol, lylal, methyl octine carbonate, eugenol, DHC, HDCL, and lilial provoked a mean response of 158, 58, 59, 33, 30, 36, and 30% of the HCA response, respectively. Similar results, following the same hierarchy between these molecules, were obtained from three different experiments, demonstrating the reproducibility of the method.

Coumarin and geraniol, postulated to be weak haptens, were not classified as contact sensitizers in the IFN $\gamma$  LLNA as they did not reach the threshold value of 15%.

#### DISCUSSION

We have previously shown, in a mouse model of fragrance allergy, that CD8 $^{+}$  T cells are effector cells of ACD to common weak allergens and are under the control of CD4 $^{+}$  T regulatory cells, which prevent their priming *in vivo*. Indeed, ACD to fragrances was obtained in CD4 $^{+}$  T-cell-depleted mice but not in normal mice. These observations suggested that mice deficient in CD4 $^{+}$  T cells could be used to detect the immunizing properties of chemicals.

In this study, we used the fragrance allergy mouse model to develop a non-radioactive LLNA-derived assay, referred to as the IFN $\gamma$  LLNA, to detect the skin-sensitizing properties of chemicals. The skin of CD4 $^{+}$  T-cell-deficient mice was painted with chemicals and the priming of CD8 $^{+}$  T cells in the draining LNs was assessed by IFN $\gamma$  production on restimulation by submitogenic doses of PHA. The rationale for using IFN $\gamma$  production as the read-out of hapten-induced T-cell priming comes from previous studies from our group, and others, that have shown that hapten-specific CD8 $^{+}$  effector T cells produce high amounts of IFN $\gamma$  (Xu *et al.*, 1996; Kehren *et al.*, 1999; Akiba *et al.*, 2002). Depletion of CD8 $^{+}$  T cells abrogated the IFN $\gamma$  production in LN cells, demonstrating that CD8 $^{+}$  T cells were the only IFN $\gamma$ -producing cell type during hapten sensitization (Kehren *et al.*, 1999; Akiba *et al.*, 2002). Therefore, the measurement of IFN $\gamma$  in draining LNs represents a direct means of studying hapten-specific effector T-cell priming. The rationale for using submitogenic doses of PHA for the detection of hapten-

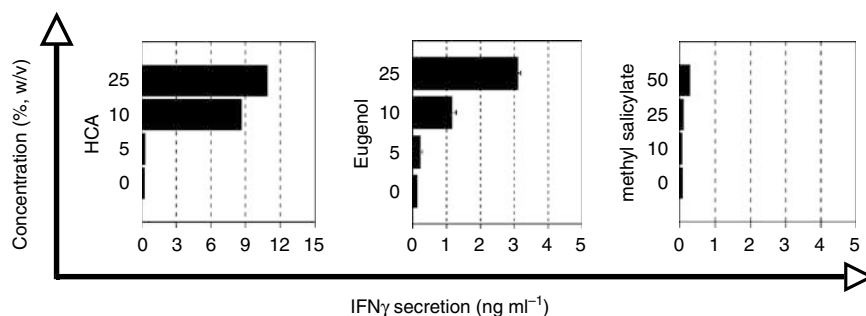


**Figure 3. Dose responses induced by different chemicals in the IFN $\gamma$  LLNA.** CD4 $^{+}$  T-cell-depleted BALB/c mice were sensitized on both ears for three consecutive days (days 0, 1, and 2) by different concentrations of chemicals or by the vehicle A/OO. At day 5, draining LN cells were collected and cultured *ex vivo* in presence of  $5 \mu\text{g ml}^{-1}$  PHA for 20 hours. IFN $\gamma$  secretion was evaluated by ELISA. Results are representative of three different experiments with four mice per group.

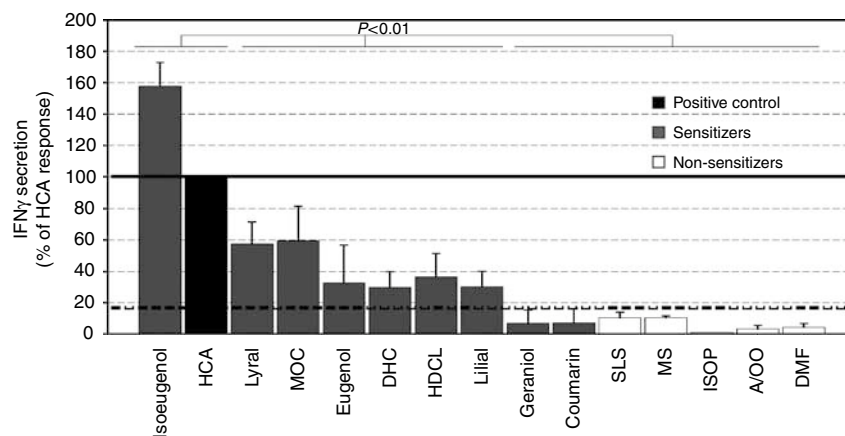


**Figure 4. Use of the IFN $\gamma$  LLNA test to detect the sensitizing properties of weak haptens.** CD4 + T-cell-depleted BALB/c mice were sensitized by different chemicals on both ears for three consecutive days (days 0, 1, and 2). All the molecules were tested at 25% (w/v) concentration, except SLS (10% (w/v)) and the vehicles A/OO and DMF. At day 5, draining LN cells were collected and cultured *ex vivo* in presence of 5  $\mu\text{g mL}^{-1}$  PHA for 20 hours. IFN $\gamma$  secretion was titrated by ELISA. IFN $\gamma$  secretion induced by the different chemicals was compared with that obtained with HCA in three different experiments, with four mice per group, and the results were expressed as mean of the percentage of the HCA response. The plain line represents the IFN $\gamma$  production induced by HCA sensitization. The dotted line represents the threshold value of 15% of the HCA response, above which results are considered positive.

specific T cells comes from previous immunological studies on antigen-specific T-cell responses. Indeed, at low doses, mitogens cannot activate resting and naïve T cells but can potentially stimulate proliferation and activation of *in vivo* pre-activated T cells (Cohen *et al.*, 1974). This protocol is particularly well suited for the study of *in vitro* T cell responses to contact allergens, as most of the weak haptens are lipophilic molecules, which cannot be incorporated into culture media for secondary T-cell proliferative responses. In this study, we monitored the IFN $\gamma$  secretion by LN cells of hapten-sensitized mice using submitogenic doses of PHA, or anti-CD3 mAb, in an *ex vivo* secondary T-cell response.

Our results show that the IFN $\gamma$  LLNA, that is production of IFN $\gamma$  by LN cells of CD4 + T-cell-deficient mice, represents a sensitive, specific, and robust assay to appreciate the sensitizing properties of chemicals. First, all tested chemicals known to be contact allergens in humans (except Geraniol and Coumarin) induced IFN $\gamma$  production in the assay. Second, none of the chemicals endowed with irritant, but not with allergenic, properties were able to trigger IFN $\gamma$  secretion by LN cells. This suggests that the IFN $\gamma$  LLNA is more specific than the radioactive LLNA, where the non-sensitizer SLS induces frequent positive reactions. The reason for the high specificity of the IFN $\gamma$  LLNA probably relies on the fact that IFN $\gamma$  production reflects activation of specific CD8 + T cells. In contrast, the radioactive LLNA measures LN cell proliferation, which may involve specific and non-specific LN cells. Third, the magnitude of IFN $\gamma$  production paralleled the sensitizing properties of chemicals. Indeed, the moderate allergens isoeugenol and HCA gave consistently higher IFN $\gamma$  production than the weak allergens eugenol, lilial, HDCL, or lyril. We report (Table S1) the EC3 values obtained for each sensitizer in a standard LLNA that serve to rank the sensitizing potency of contact allergens (Gerberick *et al.*, 2004; ICCVAM, 2008). Analysis of our results shows a good correlation between the magnitude of IFN $\gamma$  production

obtained in the IFN $\gamma$  LLNA and the EC3 values, except for DHC that gave weaker responses in the IFN $\gamma$  LLNA than in the standard LLNA.

Geraniol and coumarin were the only two weak allergens that did not induce T-cell priming in CD4 + T-cell-deficient mice. This suggests either that our assay is not sensitive enough to detect very weak haptens or that the two chemicals tested here have no sensitizing properties. In this respect, it is noteworthy that coumarin *per se* is not a contact sensitizer as we have recently reported that pure coumarin is unable to induce sensitization in a fragrance allergy mouse model (Vocanson *et al.*, 2007) and to reveal coumarin allergy in humans (Vocanson *et al.*, 2006a). In contrast, less pure coumarin preparations containing derivatives and contaminants, such as DHC, which scored positive in the IFN $\gamma$  LLNA, may promote sensitization and allergy. Whether a similar hypothesis may explain why geraniol did not induce IFN $\gamma$  production in the modified LLNA remains to be tested.

IFN $\gamma$  production was already used as an endpoint to screen for sensitizers in LLNA-derived protocols in normal mice (Dearman *et al.*, 1999; Azam *et al.*, 2005). However, the investigators gave low credit to this strategy because the results showed poor sensitivity (Dearman *et al.*, 1999) and high variability (Azam *et al.*, 2005). The poor sensitivity was probably related to the presence, in normal mice, of CD4 + T cells with suppressive activity that prevented the priming of and IFN $\gamma$  production by CD8 + effector T cells. Indeed, as shown by the present study, depletion of CD4 + T cells before sensitization dramatically increased IFN $\gamma$  production leading to a very sensitive IFN $\gamma$  LLNA (Figure 1). Similarly to the results recorded by Azam *et al.* (2005), we observed that the magnitude of IFN $\gamma$  secretion in the test varied greatly from one experiment to another. However, to avoid the negative impact of this variability for a potency-based classification of sensitizers, we introduced a positive control in each experiment and the results recorded for test haptens were

expressed as a percentage of the positive control response. Interestingly, in that experimental setting, repetition of the experiments always led to similar categorization of chemicals, indicating that the IFN $\gamma$  LLNA is not only useful for detecting contact allergens but is robust enough to assess and classify their respective sensitizing potency.

In conclusion, we demonstrate here that the IFN $\gamma$  LLNA accounts for a promising non-radioactive alternative test to detect immune responses induced by common chemicals endowed with skin sensitizing properties. Our results need to be validated with other sensitizers and by inter-laboratory experiments, notably to determine a precise threshold value that could discriminate between weak allergens and non-sensitizers.

## MATERIALS AND METHODS

### Mice

Female BALB/c mice were purchased from Charles River Laboratories (L'Arbresle, France). Animals were left to acclimatize before entering the study and were provided with food and water *ad libidum*. All the mice were used between 8 and 10 weeks of age. Experimental procedures were approved by the institutional review board on animal experiments (Comité Régional d'Ethique sur l'Expérimentation Animale).

### Chemicals

DHC (98% pure), geraniol (98% pure),  $\alpha$ -HCA (85% pure), HDCL (95% pure), isoeugenol (98% pure), SLS (99% pure), and MS (99% pure) were purchased from Sigma-Aldrich (Saint Quentin en Fallavier, France). Eugenol, lylal, lilial, and MOC were purchased from Firmenich (Neuilly sur Seine, France) and ISOP (99.7% pure) from Merck. Coumarin (Rhodiascent extra pure, >99.9% pure) was produced in Rhodia Organics (Lyon, France). All the chemicals were prepared in A/OO (Carlos Erba and Lessieur) (4:1, v/v) freshly before application, except for SLS, DHC, and coumarin, which were dissolved in DMF (Sigma). Chemicals were dispensed with gloves for dermal protection.

### Antibody depletion of CD4<sup>+</sup> T cells *in vivo*

The rat anti-mouse CD4 mAb GK 1.5 was obtained from ATCC (Rockville, MD). Hybridomas were grown as ascites in pristane-primed Swiss-nude mice. Mice were given i.p. injections of 20  $\mu$ g of anti-CD4 mAbs in 200  $\mu$ l on days -1, 0, 1, and 4. Depletions >98% were observed in all the experiments (Figure S1).

### Sensitization of mice

The sensitization procedure was conducted according to the standard LLNA protocol (Kimber and Dearman, 1991; OECD and Chemicals GfT, April 2002). Briefly, groups of BALB/c mice ( $n = 3-4$ ) were painted by topical application of 25  $\mu$ l per ear of the appropriate dilution of the test substance, or of vehicle alone, on the dorsum of both ears, daily for three consecutive days (days 0, 1, and 2).

### Preparation of draining LN cells

The days following chemical application, draining auricular LNs were collected in complete RPMI medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine,

100  $\mu$ g ml<sup>-1</sup> streptomycin, 100 U ml<sup>-1</sup> ampicillin and  $5 \times 10^{-5}$  M of  $\beta$ -2 mercaptoethanol) and pooled per experimental group. A single-cell suspension of LN cells was prepared by mechanical disaggregation through a 100  $\mu$ m nylon filter (Becton Dickinson, Le Pont de Claix, France). Viable cell counts were carried out by exclusion of 0.5% trypan blue and dilutions of LN cells were prepared in complete RPMI medium.

### Analysis of chemical-induced T-cell responses

**IFN $\gamma$ -ELISPOT.** The number of IFN $\gamma$ -producing cells in the draining LNs of mice sensitized by chemicals was determined using an ELISPOT assay. Briefly, 96-well nitrocellulose plates (MAIP N4510; Millipore, Saint Quentin-en-Yvelines, France) were wet with 70% ethanol for 10 minutes, washed in phosphate buffer saline and coated overnight at 4 °C with an anti-IFN $\gamma$  antibody (R46A2; BD-Pharmingen, Le Pont de Claix, France). The following day, the plates were blocked with phosphate buffer saline/1% bovine serum albumin for 2 hours at 37 °C, and they were washed five times with phosphate buffer saline/Tween 0.05% before use. Auricular LN cells were then dispensed in the plates ( $10^5$  per well) and incubated in presence of phytohemagglutinin-P (PHA-P; Sigma) overnight at 37 °C, 5% CO<sub>2</sub>. After washing, plates were incubated 2 hours at 37 °C, 5% CO<sub>2</sub> with a biotinylated anti-IFN $\gamma$  antibody (XMG1.2; BD-Pharmingen). IFN $\gamma$  SFCs were developed using Avidin Horse Radish Peroxydase (CliniSciences, Montrouge, France) incubated for 20 minutes at room temperature, and extensively washed before adding the substrate (aminoethyl carbazole; Sigma). The number of IFN $\gamma$  SFCs present in each well was counted using the Carl Zeiss vision ELISPOT (Carl Zeiss Vision, D-85399, Hallberghoos, Germany) and the results were expressed as IFN $\gamma$  SFCs/ $10^5$  LN cells.

**IFN $\gamma$ -ELISA.** Secretion of IFN $\gamma$ -producing cells in the draining LNs of mice sensitized by chemicals was determined by ELISA. A total of 2 million LN cells ml<sup>-1</sup> were cultured, in duplicate or triplicate, in 96-well plates in RPMI complete medium, in the presence of PHA-P, or in the presence of an anti-CD3-mAb (2c11; BD-Pharmingen), at 37 °C, 5% CO<sub>2</sub>. Of note, the same batch of PHA was used for all the experiments. Cell-free supernatants were harvested after 16-48 hours, and IFN $\gamma$  production was titrated by ELISA using the R&D System (Lille, France) DuoSet kit following the manufactural protocol.

### Statistics

Statistics were performed using a one-way analysis of variance test to compare groups of chemicals.  $P < 0.01\%$  was used as the level of significance.

### CONFLICT OF INTEREST

The authors state no conflict of interest.

### ACKNOWLEDGMENTS

We thank David Basketter for providing LLNA data resources. We also thank Delphine De Queral for statistical analysis. This work was supported by institutional grants from Université Claude Bernard Lyon 1 and INSERM.

### SUPPLEMENTARY MATERIAL

**Figure S1.** *In vivo* treatment of BALB/c mice with anti-CD4 mAb leads to profound CD4<sup>+</sup> T-cell depletion in lymphoid organs.

**Figure S2.** Development of the IFN $\gamma$  LLNA in CD4+ T-cell-depleted mice. Anti-CD3 mAb restimulation.

**Figure S3.** Detection of chemical-induced T-cell responses by IFN $\gamma$ -ELISA and IFN $\gamma$ -ELISPOT techniques.

**Table S1.** LLNA EC3 values (from the NICEATM database) and sensitizing potency for the test chemicals.

## REFERENCES

- Akiba H, Kehren J, Ducluzeau MT, Krasteva M, Horand F, Kaiserlian D *et al.* (2002) Skin inflammation during contact hypersensitivity is mediated by early recruitment of CD8+ T cytotoxic 1 cells inducing keratinocyte apoptosis. *J Immunol* 168:3079–87
- Azam P, Peiffer JL, Ourlin JC, Bonnet PA, Tissier MH, Vian L *et al.* (2005) Qualitative and quantitative evaluation of a local lymph node assay based on *ex vivo* interleukin-2 production. *Toxicology* 206:285–98
- Blauvelt A, Hwang ST, Udey MC (2003) 11. Allergic and immunologic diseases of the skin. *J Allergy Clin Immunol* 111:S560–70
- Coenraads PJ, Goncalo M (2007) Skin diseases with high public health impact. *Contact dermatitis. Eur J Dermatol* 17:564–5
- Cohen MH, Chretien PB, Felix EL, Loyd BC, Ketcham AS, Albright LA *et al.* (1974) Augmentation of lymphocyte reactivity in guinea pigs, mice, monkeys and humans sensitised to BCG, dinitrochlorobenzene or nitrogen mustard. *Nature* 249:656–8
- Dearman RJ, Hilton J, Basketter DA, Kimber I (1999) Cytokine endpoints for the local lymph node assay: consideration of interferon-gamma and interleukin 12. *J Appl Toxicol* 19:149–55
- Dearman RJ, Wright ZM, Basketter DA, Ryan CA, Gerberick GF, Kimber I (2001) The suitability of hexyl cinnamic aldehyde as a calibrant for the murine local lymph node assay. *Contact Dermatitis* 44:357–61
- Dubois B, Chapat L, Goubier A, Kaiserlian D (2003) CD4+CD25+ T cells as key regulators of immune responses. *Eur J Dermatol* 13:111–6
- Gerberick GF, Ryan CA, Kern PS, Dearman RJ, Kimber I, Patlewicz GY *et al.* (2004) A chemical dataset for evaluation of alternative approaches to skin-sensitization testing. *Contact Dermatitis* 50:274–88
- ICCVAM (2008) *Draft ICCVAM Background Review Document: Use of the Murine Local Lymph Node Assay (LLNA) to Determine Skin Sensitization potency Categories*. Research Triangle Park, NC: National Toxicology Program. Available <http://iccvam.niehs.nih.gov/methods/immunotox/llna-BRD.htm>
- Kehren J, Desvignes C, Krasteva M, Ducluzeau MT, Assossou O, Horand F *et al.* (1999) Cytotoxicity is mandatory for CD8(+) T cell-mediated contact hypersensitivity. *J Exp Med* 189:779–86
- Kimber I, Dearman RJ (1991) Investigation of lymph node cell proliferation as a possible immunological correlate of contact sensitizing potential. *Food Chem Toxicol* 29:125–9
- Kimber I, Dearman RJ, Basketter DA, Ryan CA, Gerberick GF (2002) The local lymph node assay: past, present and future. *Contact Dermatitis* 47:315–28
- Martin SF (2004) T lymphocyte-mediated immune responses to chemical haptens and metal ions: implications for allergic and autoimmune disease. *Int Arch Allergy Immunol* 134:186–98
- Maurer T (2007) Guinea pigs in hypersensitivity testing. *Methods* 41: 48–53
- OECD and Chemicals GfT (April 2002) Draft New Guideline 429 “skin sensitization: Local Lymph Node Assay”
- Saint-Mezard P, Berard F, Dubois B, Kaiserlian D, Nicolas JF (2004a) The role of CD4+ and CD8+ T cells in contact hypersensitivity and allergic contact dermatitis. *Eur J Dermatol* 14:131–8
- Saint-Mezard P, Rosieres A, Krasteva M, Berard F, Dubois B, Kaiserlian D *et al.* (2004b) Allergic contact dermatitis. *Eur J Dermatol* 14: 284–295
- Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z *et al.* (2006) Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 212:8–27
- Trautmann A, Akdis M, Schmid-Grendelmeier P, Disch R, Brocker EB, Blaser K *et al.* (2001) Targeting keratinocyte apoptosis in the treatment of atopic dermatitis and allergic contact dermatitis. *J Allergy Clin Immunol* 108:839–46
- Vocanson M, Goujon C, Chabeau G, Castelain M, Valeyrie M, Floc’h F *et al.* (2006a) The skin allergenic properties of chemicals may depend on contaminants—evidence from studies on coumarin. *Int Arch Allergy Immunol* 140:231–8
- Vocanson M, Hennino A, Cluzel-Tailhardat M, Saint-Mezard P, Benetiere J, Chavagnac C *et al.* (2006b) CD8+ T cells are effector cells of contact dermatitis to common skin allergens in mice. *J Invest Dermatol* 126:815–20
- Vocanson M, Valeyrie M, Rozieres A, Hennino A, Floc’h F, Gard A *et al.* (2007) Lack of evidence for allergenic properties of coumarin in a fragrance allergy mouse model. *Contact Dermatitis* 57:361–4
- Xu H, DiIulio NA, Fairchild RL (1996) T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: interferon gamma-producing (Tc1) effector CD8+ T cells and interleukin (Il) 4/Il-10-producing (Th2) negative regulatory CD4+ T cells. *J Exp Med* 183:1001–12

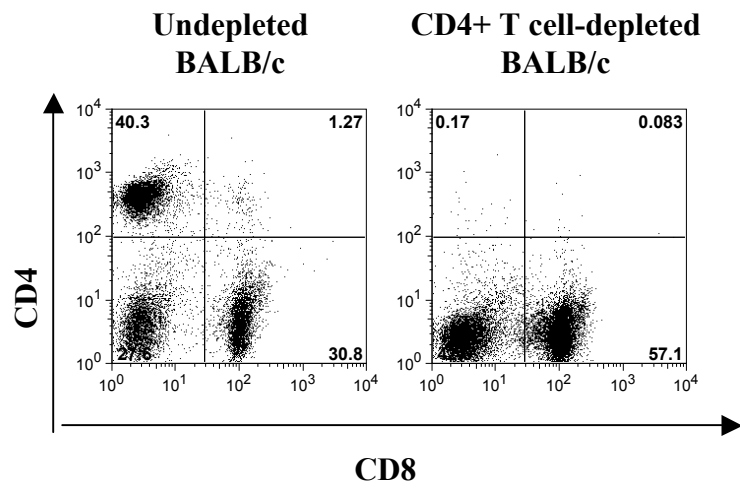
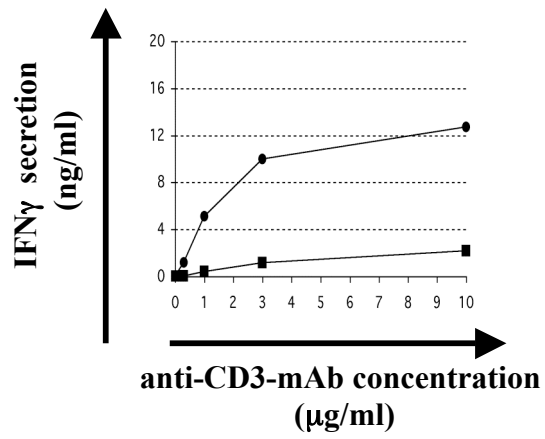


**Figure S1.**

A



B

| PHA concentration<br>( $\mu\text{g/ml}$ ) | IFN $\gamma$ secretion<br>(ng/ml) |      | Stimulation Indices<br>(SI) |
|-------------------------------------------|-----------------------------------|------|-----------------------------|
|                                           | HCA                               | A/OO |                             |
| 0.3                                       | 1.18                              | 0.03 | 39                          |
| 1                                         | 5.10                              | 0.41 | 12                          |
| 3                                         | 9.98                              | 1.15 | 9                           |
| 10                                        | 12.70                             | 2.18 | 6                           |

C

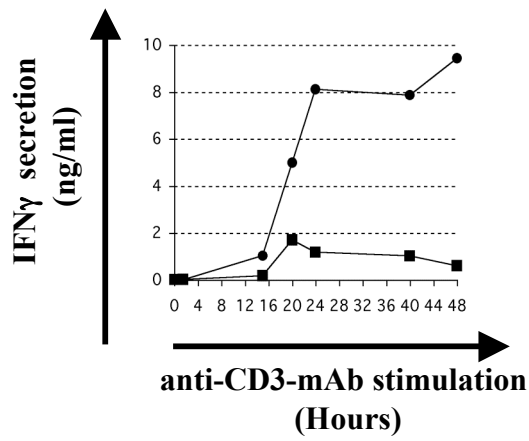


Figure S2.

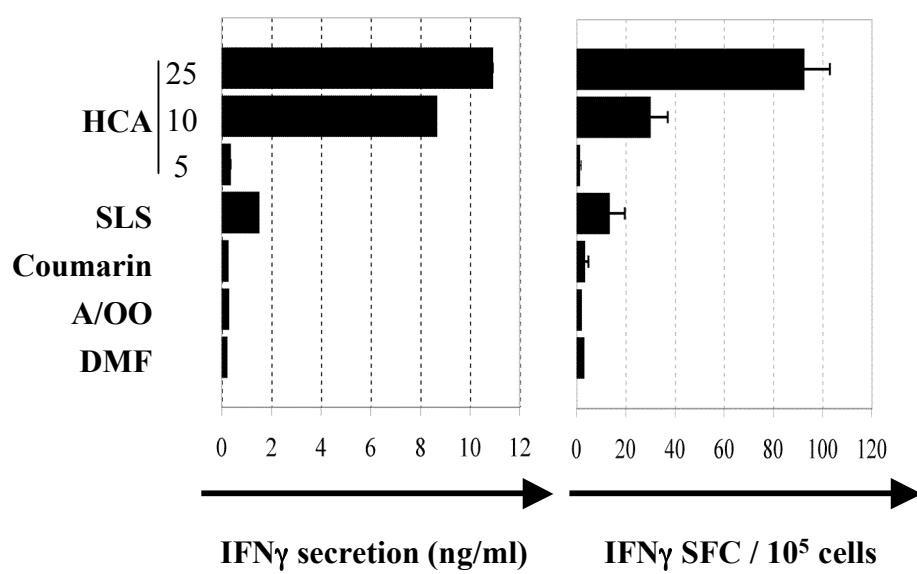


**Figure S3.**

| <b>Chemicals</b>  | <b>Vehicle</b> | <b>EC3 Value (%)</b> | <b>Number of experiments</b> | <b>Lowest - Highest EC3 values (%)</b> | <b>Sensitizing potency</b> |
|-------------------|----------------|----------------------|------------------------------|----------------------------------------|----------------------------|
| <b>Isoeugenol</b> | A/OO           | 1.6*                 | 28                           | 0.5 - 3.3                              | Moderate                   |
| <b>HCA</b>        | A/OO           | 5.5*                 | 19                           | 0.04 - 14.7                            |                            |
|                   | DMF            | nd                   | nd                           | nd                                     |                            |
| <b>DHC</b>        | DMF            | 3.3                  | 1                            | -                                      |                            |
| <b>MOC</b>        | A/OO           | -                    | -                            | -                                      | Weak                       |
| <b>Eugenol</b>    | A/OO           | 10.4*                | 16                           | 4.9 - 20.4                             |                            |
| <b>Lylal</b>      | A/OO           | 17                   | 1                            | -                                      |                            |
| <b>Lilial</b>     | A/OO           | 18.8*                | 2                            | 18.7 - 19                              |                            |
| <b>HDCL</b>       | A/OO           | 23.2*                | 6                            | 18 - 33                                |                            |
| <b>Geraniol</b>   | A/OO           | 34.1*                | 2                            | 20.4 - 57                              |                            |
| <b>Coumarin</b>   | DMF            | nc                   | 5                            | -                                      |                            |
| <b>SLS</b>        | DMF            | 4.7*                 | 6                            | 1.5 - 17.1                             | False positive             |
| <b>MS</b>         | A/OO           | nc                   | 1                            | -                                      | Non-sensitizers            |
| <b>ISOP</b>       | A/OO           | nc                   | 1                            | -                                      |                            |

**Table S1.**