

An intravenous exposure mouse model for prediction of potential drug-sensitization using reporter antigens popliteal lymph node assay

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ABSTRACT: Immune-mediated drug hypersensitivity is a particularly concerning health-safety issue among clinicians given its unpredictability and potentially life-threatening effects, especially with exposure to intravenous drugs. Therefore, the development of intravenous drug-exposure models for drug-hazard assessments has garnered increasing interest in recent years. In this study, we used reporter antigens popliteal lymph node assay to investigate the potential value of intravenous exposure to a selected variety of allergenic compounds, including ovalbumin (OVA), concanavalin A (ConA) and diclofenac. The trinitrophenyl (TNP)-specific antibody-forming cells were used to assess the systemic immune responses to a bystander antigen. Mice were subsequently sensitized by TNP-OVA, and then intravenous exposure to one of the selective compounds. As expected, all positive compounds induced significant popliteal lymph node (PLN) proliferation compared with the control. OVA significantly increased Cluster of Differentiation 4 receptors (CD⁴)⁺ interleukin-4 (IL-4)⁺ T-helper 2 (Th2) cells and, consequently, increased the ratios of IL-4/interferon- γ (IFN- γ) antibody-forming cells (AFCs) in PLNs, while bringing about a dose-dependent increase in immunoglobulin G1 (IgG1) AFCs; these findings indicate that a Th2 hypersensitivity response was induced. A Th2 response was also observed in diclofenac sodium-treated groups, and for ConA, a more mixed Th1/Th2 immune response appeared to be induced. In addition, there was no marked reaction with the negative compound. Together, it seems likely that the intravenous exposure model may be useful for drug-induced systemic hypersensitivity assessments. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: immune-mediated drug hypersensitivity reaction; intravenous; mouse model; reporter antigen; popliteal lymph node assay

INTRODUCTION

Immune-mediated drug hypersensitivity reaction (IDHR), although constituting only a minority (20%) of adverse drug reactions (Gruchalla, 2001; Schnyder and Pichler, 2009), is a serious clinical problem that frequently causes serious, unpredictable systemic reactions and may even result in death. In China, traditional Chinese medicine injectables (TCMIs) have been widely used in patients suffering from cardiovascular diseases and cancer for over 60 years; however, IDHRs induced by TCMIs has been reported to have increased significantly over the past 10 years (Ko, 2004). Furthermore, the mechanisms of IDHR are still inadequately understood, with regard to their multi-factorial complexity, including drug administration, genetic constitution, age and gender (Simons *et al.*, 2007). Consequently, to date, there is a lack of reliable assessments that can be used to identify IDHR preclinically, especially in drug-induced hypersensitivity identification for intravenously administered drugs. Therefore, there is a great need to establish an intravenous exposure model for drug-induced hypersensitivity potential identification, especially for TCMIs.

Popliteal lymph node assay (PLNA), a straightforward animal test, has been recognized as a simple tool that is a reliable, fast and reproducible method for IDHR assessment (Carey *et al.*, 2006; Gehrs and Smialowicz, 1999; Gutting *et al.*, 2002b; Kammuller *et al.*, 1989; Lovik *et al.*, 2007; Nierkens *et al.*, 2005a). Since 1976, PLNA has been used to study graft vs host reactions, initially by Gleichmann and Gleichmann, and studies

have demonstrated that numerous drugs and chemicals known to induce IDHR in humans, such as penicillin, diclofenac and cadmium, cause PLN reactions following injection into the footpad (Aida *et al.*, 1998; Carey *et al.*, 2006; Gleichmann and Gleichmann, 1976; Gutting *et al.*, 2002b; Samuelsen *et al.*, 2008); the results of these tests showed close correlations.

PLNA can be classified into direct, secondary, adoptive, reporter antigens and adjuvant PLNAs. Of all modifications, reporter antigens PLNA (RA-PLNA) has greatly compensated for the limitations of direct PLNA in providing insight into the mechanism (Albers *et al.*, 1997; Pieters and Albers, 1999). In RA-PLNA, levels of RA-specific antibody-forming cells (AFCs) are measured to analyze T-cell-dependent immune activation. The activation of CD⁴⁺ T helper-1 cells may be identified as markers of the induction of IgG2a and/or IgG2b AFCs, whereas CD⁴⁺ T helper-2 cells may manifest the induction of immunoglobulin E (IgE) and/or IgG1 AFCs (Albers *et al.*, 1997; Gutting *et al.*, 2002b; Isola *et al.*, 2008). In addition, efficient immune responses to RA

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need cognate T, B cell interactions and co-stimulatory adjuvant signals (Nierkens *et al.*, 2002). Therefore, any sensitized drug or chemical may induce formation of RA-specific AFCs. Thus, the RA-PLNA may well identify the RA-specific T-cell-dependent immune-sensitized potential of drugs and chemicals injected into footpads.

Although there are several advantages of RA-PLNA in drug-immunosensitizing potential assessment, models for drugs that are injected subcutaneously in the footpad have been acutely deficient, while most drugs are administered orally or intravenously. Fortunately, an oral exposure model for IDHR prediction by RA-PLNA has been demonstrated to be useful (Nierkens *et al.*, 2005b). However, there is as yet no intravenous exposure model that has been developed for hazard assessments of drugs that are administered intravenously. Therefore, in the present study, we evaluated whether the intravenous administration of drugs inducing IDHR might stimulate systemic immunosensitization by RA-PLNA. In these experiments, a T-cell-dependent antigen, trinitrophenyl-ovalbumin (TNP-OVA) was used as RA, subcutaneously injected into footpads for sensitization on day 1. Mice were then intravenously injected with different positive or negative compounds in multiple doses. For the complex constituents of TCMI, including proteins and low-molecular-weight compounds (LMWC), in order to validate the intravenous model, two positive protein antigens (OVA and concanavalin A, ConA) associated with human IDHR (Dearman *et al.*, 2009b; Gleichmann and Gleichmann, 1976; Valdimarsson and Gross, 1973), and a low-molecular-weight compound (diclofenac sodium, DF) that had been demonstrated to induce IDHR in mice (Gutting *et al.*, 2002b) were used as positive compounds, and phenobarbital, since it failed to induce any PLN response, was used as negative control compound. PLN weight index and cellularity were determined in this study, and anti-TNP-specific IgM, IgE, IgG1, IgG2a and IgG2b AFCs were also quantified as outcomes in tests for each group, as well as lymphocyte phenotype (CD^{3+} , CD^{4+} , CD^{8+} and CD^{19+}) and intracellular cytokines (IL-4 and IFN- γ) in isolated PLNs, being determined by flow cytometry.

MATERIALS AND METHODS

Animals

Young adult (6- to 8-week-old, specific-pathogen-free) female BALB/c strain mice (Barrier Animal Breeding Unit, Experimental Animal Department, Huazhong University of Science and Technology, Wuhan, China) were used throughout these studies. Food and water were available *ad libitum*. The diet used was Special Diet Services Rat and Mouse no. 1 Maintenance Diet, comprising primarily cereal products. The mice were housed in plastic cages and maintained at $23 \pm 3^\circ\text{C}$; the relative humidity was $50 \pm 5\%$ with a 12:12 h light-dark cycle. All experiments were conducted in accordance with guidelines prescribed by the Animal Experiments Committee of the College of Pharmaceutical Sciences, the Zhejiang University (Hangzhou, China).

Chemicals and Reagents

Ovalbumin and ConA were procured from Sigma-Aldrich, phenobarbital sodium (PB) from Toronto Research Chemicals Inc. and DF from the National Institute for the Control of Pharmaceutical and Biological products. MultiScreenHTS Filter Plates for immunoblot were purchased from Millipore Inc.

Alkaline phosphatase-conjugated goat anti-mouse IgM, IgG1, IgE, IgG2a and IgG2b were purchased from the SouthernBiotech Inc. Antibodies (mouse CD^{3+} , CD^{4+} , CD^{8+} , CD^{19+}) used in flow cytometry were obtained from eBiosciences Inc. The conjugates TNP-BSA (TNP²⁶-bovine serum albumin) and TNP-OVA (TNP¹³-ovalbumin) were obtained from the Biosearchtech Inc. The BCIP/NBT alkaline phosphatase substrate kit IV was purchased from VectaMount Inc.

Intravenous Exposure Models

On day 1, animals received an injection into both hind footpads in a heel-to-toe direction. Except for the normal sodium (NS) group, each group was injected with TNP-OVA (10 μg) in 50 μl of NS. On days 3, 5 and 7, each treatment group received an injection into the tail vein with different concentrations of compounds in NS; the NS group and TNP-OVA control animals received only the vehicle in the same volume. Furthermore, on day 9, each treatment group (including TNP-OVA control) animal was challenged again with TNP-OVA (10 μg) in 50 μl of NS injected into footpads. On day 11, mice were sacrificed by cervical dislocation, and the draining PLNs were extracted; PLNs were then placed in phosphate buffered solution (PBS) (containing 1% BSA) and freed of excess fatty tissue.

Determination of PLN Weight Indexes and Cell Numbers

The weight of PLNs was determined in each mouse, and the weight index (index = weight of PLNs/mouse body weight) was calculated. Then, the PLNs were ruptured with a 26-gage needle and the cells were extracted by gentle agitation, washed with 1% BSA-PBS by centrifugation, re-suspended in 1% BSA-PBS and counted with a Coulter Counter, then the lymph node cell numbers were presented for each PLN.

ELISPOT Assay for TNP-specific AFCs

The ELISPOT assay was performed using a protocol similar to that described previously (Schlielen *et al.*, 1995). An Immobilon[®]-P bottom plate was pre-coated overnight at 4°C with TNP-BSA (10 $\mu\text{g ml}^{-1}$) in PBS. Aliquots of 50 μl , from the cell suspension ($1 \times 10^6 \text{ ml}^{-1}$), were placed in one well of the 96-well Immobilon[®]-P bottom plate. The cells were incubated at 37°C for 4 h. Thereafter, the plates were washed to remove cell debris, and membranes were incubated for 18 h at 4°C with diluted alkaline phosphatase-conjugated goat anti-mouse IgM (1:5000), IgG1 (1:1000), IgE (1:500), IgG2a (1:2500) or IgG2b (1:2500) in 1% BSA-PBS. After PBS and Tween-20 washing, wells were incubated with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)-nitro blue tetrazolium (NBT) substrate solution to visualize TNP-specific antibody spots. Then the membranes were dried and the numbers of IgM, IgG1, IgG2b, IgG2a or IgE AFCs were determined using an immunospot detector (Immunospot IV, CTL, USA). AFCs were reported as the number of AFCs per 10^6 PLN cells. PLN cell numbers that were determined previously were used to calculate the number of AFCs per 10^6 recovered PLN cells.

Flow Cytometry for Cell Subtypes and Intracellular Cytokines

For cell surface-subtype staining, a total of 2×10^5 PLN cells from each animal were prepared and re-suspended in specific

PE-Cy7-conjugated monoclonal antibodies against CD¹⁹, PE-Cy5-conjugated monoclonal antibodies against CD³, FITC-conjugated monoclonal antibodies against CD⁴, PE-conjugated monoclonal antibodies against CD⁸ or isotype controls at a concentration of 1:100 (v/v) in 1% BSA-PBS and incubated in the dark for 30 min at 4°C. These were then washed with PBS and immediately analyzed on an LSR-II instrument (Becton Dickinson). A minimum of 8000 cells were analyzed for each sample.

For intracellular cytokines staining, 1×10^6 PLN cells from each animal were prepared and stained for surface subtypes with PE-Cy5-conjugated monoclonal antibodies against CD3 and FITC-conjugated monoclonal antibodies against CD8, following the protocol described previously. Thereafter, standard fixation/permeabilization protocols, described in the eBioscience fixation/permeabilization kit, were conducted. Finally, antibodies (APC-conjugated anti-IL-4 and PE-Cy7-conjugated anti-IFN- γ) for intracellular cytokine detection were added in recommended quantities and incubated in the dark at 4°C for 30 min. Then they were washed with PBS and immediately analyzed on an LSR-II instrument (Becton Dickinson).

Statistics

Statistical analyses were performed with SPSS11.5 Statistical software (SPSS Inc). All data were expressed as means $\pm$ SEM, and analyzed using the Student's *t*-test for comparisons between two groups and one-way ANOVA for multiple groups; this was followed by Fisher's least significant difference (LSD) test. Statistically significant differences were considered for values with * $P < 0.05$ and ** $P < 0.01$.

RESULTS

PLN Weight Indexes and Cell Numbers

Cell proliferation in PLNs was demonstrated by PLN weight indexes (Fig. 1A) and cell numbers (Fig. 1B). Following sensitization with a low dose of TNP-OVA, levels of PLN weight indexes and cell numbers in control animals were not significantly different from those in NS animals. Compared with the control, all positive compounds in specific concentrations resulted in significant enhancement in weight indexes, with the exception of OVA at 10 mg kg⁻¹. In addition, cell numbers were significantly increased by all compounds. Furthermore, OVA induced a dose-dependent reaction both in PLN weight indexes and cell numbers. In contrast, PB, as a negative compound, failed to induce any significant increase in PLN weight indexes and cellularity.

TNP-specific AFC Numbers

The TNP-specific AFCs were detected using an Elispot assay. An anti-TNP-specific positive spot in the Elispot plate appeared as circular, densely granulated foci in contrast to a negative one with few or no spots (Fig. 2A). Compared with the NS, mice that were challenged with TNP-OVA only in the footpad on days 1 and 9 had slightly increased TNP-specific antibody levels in the control group, but no statistically significant differences were observed ($P > 0.05$). The IgM AFC numbers were significantly increased in the 40 mg kg⁻¹ dose of OVA group and all dose groups of ConA (Fig. 2B). Only mice in the ConA 10 and 20 mg kg⁻¹ dose groups manifested significantly increased

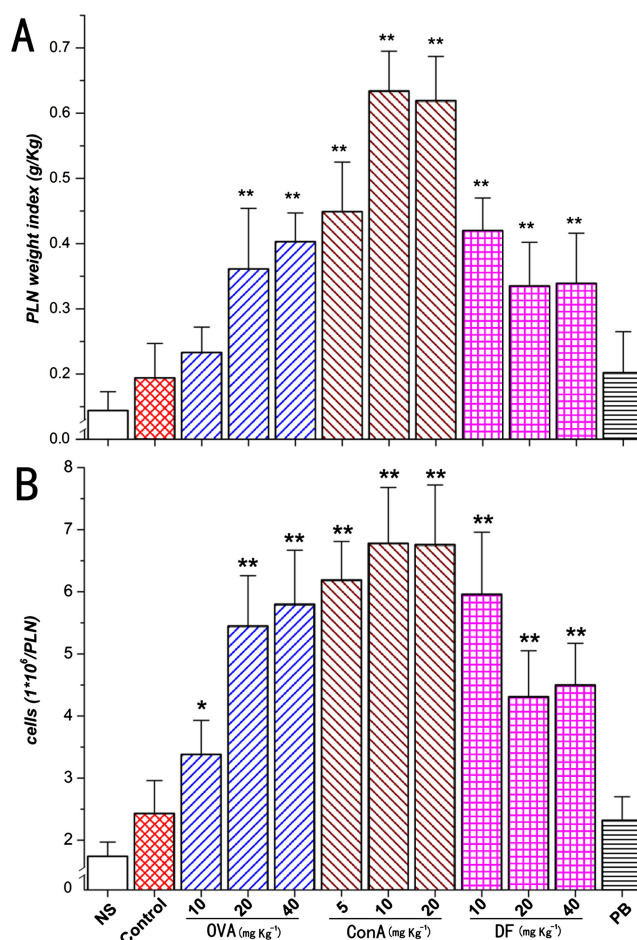


Figure 1. Weight indexes and total cell numbers presented in PLNs of intravenously treated mice. On Day 11, PLNs were isolated and weights were determined for weight-index calculation and, cell numbers were counted. Bars represent group means $\pm$ SEM of 6–8 mice per group. Statistically significant differences are indicated by * $p < 0.05$ and ** $p < 0.01$ when compared with the control.

TNP-specific IgE levels (Fig. 2C). With the exception of OVA in the 10 mg kg⁻¹ dose and DF 20 mg kg⁻¹ dose groups, which had only moderately enhanced TNP-specific IgG1, AFC numbers in all other positive compound groups were significantly increased and dose-dependently increased in OVA-treated groups (Fig. 2D). In addition, IgG2a and IgG2b AFCs were also determined for T-helper-1 cell assessments; however, only ConA in 10 and 20 mg kg⁻¹ groups demonstrated significant increases in the two AFCs (Fig. 2E, F). No marked reactions were observed in the PB group.

Lymphocyte Subpopulation Analysis

Four-color flow cytometry was used to analyze the proportions of the major lymphocyte subpopulations in PLNs in NS, control, 20 mg kg⁻¹ OVA, 10 mg kg⁻¹ ConA and 20 mg kg⁻¹ DF groups. Representative surface-staining scatter-plots are presented in Fig. 3(A). Compared with the NS group, levels of all lymphocyte subpopulations were induced without significant differences (data not shown). As shown in Fig. 3(B), compared with the control, all positive compounds induced significant growth of

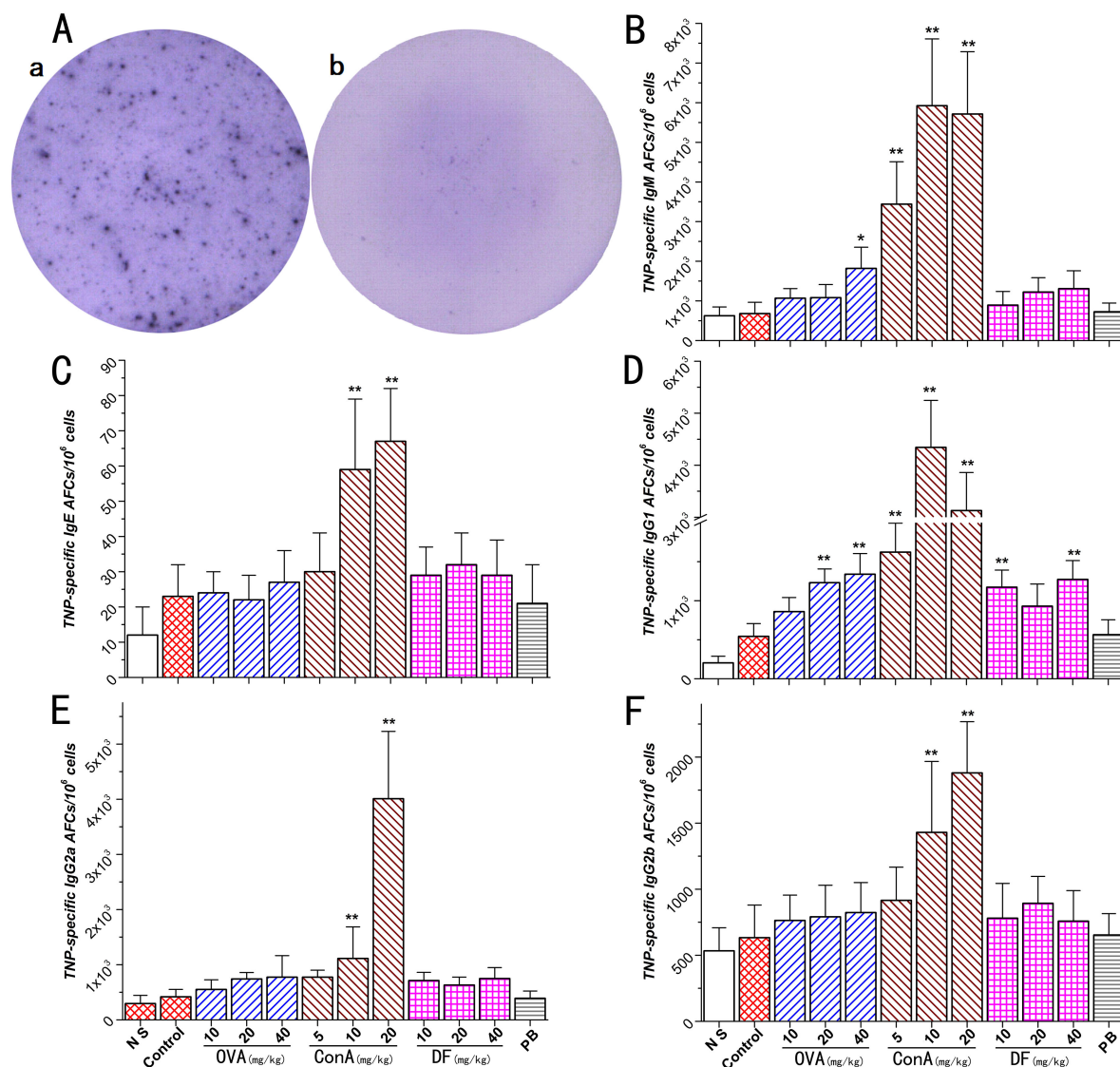


Figure 2. TNP-specific AFCs in PLNs in response to specific doses of selective compounds in intravenously treated mice. On Day 11, PLNs were isolated and TNP-specific IgM (B), IgE (C), IgG1 (D), IgG2a (E) and IgG2b (F) were measured using the Elispot assay. Anti-TNP-specific positive spots appeared to be circular, densely granulated foci (A-a), negative interactions, in contrast, were produced lightly or marked by the absence of spots (A-b). Bars represent AFCs/10⁶ PLN cells (mean $\pm$ SEM) of 6–8 mice per group. *Statistically significant differences ($p < 0.05$) and **statistically significant differences ($p < 0.01$) refer to the corresponding control.

the CD¹⁹⁺ B cell subpopulations, and only ConA induced significant differences in CD³⁺ T cell numbers. OVA and ConA significantly increased numbers of CD³⁺CD⁴⁺ T cells in the PLNs, but there was no significant difference in the DF group, although numbers in this group were also slightly increased. The only significant effect of increased CD³⁺CD⁸⁺ T-cell subpopulations was observed in mice treated with ConA.

Intracellular Cytokine Measurements

Intracellular IL-4 and IFN- γ inductions were studied to determine T-helper 1/2 (Th1/Th2) differentiations in CD⁴⁺T cells using four-color flow cytometry. A CD³⁺ and CD⁸⁺ negative gating was used to analyze CD⁴⁺ T-cell subpopulations. Representative scatter plots for gating analysis were presented in Fig. 4(A). Percentages of intracellular cytokine cells and their ratios are shown in Fig. 4(B). Percentages of CD⁴⁺ and IL-4⁺ T cells in PLNs

were significantly increased following intravenous injection of 20 mg kg⁻¹ OVA or 10 mg kg⁻¹ ConA when compared with the control. The number of CD⁴⁺ and IFN- γ ⁺ T cells was also increased after ConA injection, but the ratio of IL-4/IFN- γ showed no significant differences. Although intravenous injection of 20 mg kg⁻¹ of DF did not significantly increase the percentage of CD⁴⁺ and IL-4⁺ T cells in PLNs, it significantly enhanced the ratio of IL-4/IFN- γ .

DISCUSSION

It is known that approximately 20% of adverse drug reactions can be attributed to IDHR, which is a serious clinical problem (Gruchalla, 2001; Schnyder and Pichler, 2009). In China, TCMI are widely available in the market and have played an important clinical role for decades. However, owing to the complex constituents, IDHR induced by TCMI has become increasing

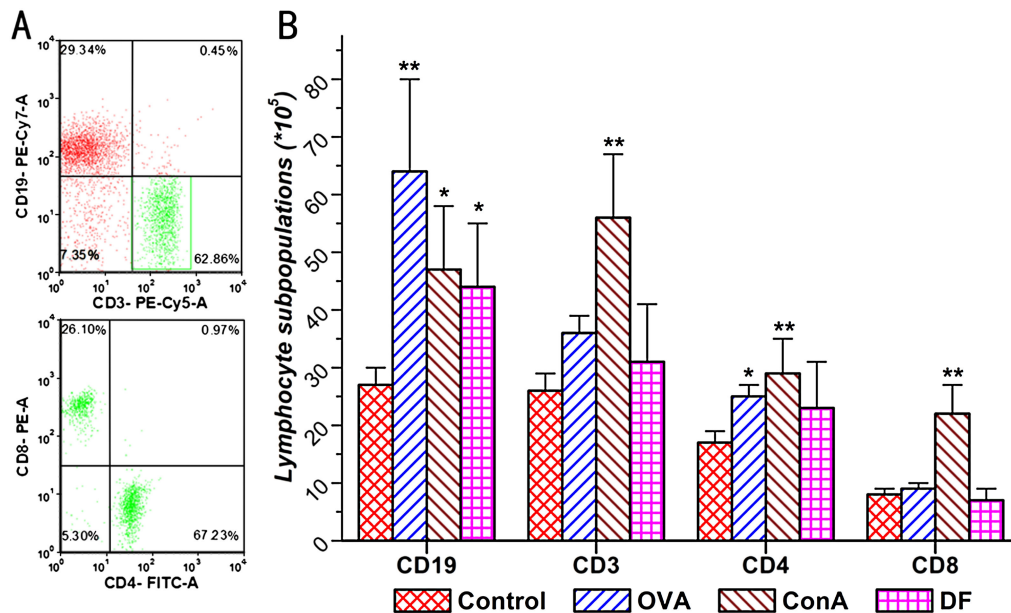


Figure 3. Proportions of lymphocyte subpopulations in PLNs. The percentages of lymphocyte subpopulations were assessed by four-color flow cytometry (A) and then converted into the total cell numbers of cells of each subpopulation in each mouse (B). Each value represents the mean $\pm$ SEM of 4-6 mice. Statistically significant differences are indicated by * $p < 0.05$ and ** $p < 0.01$ compared with the control.

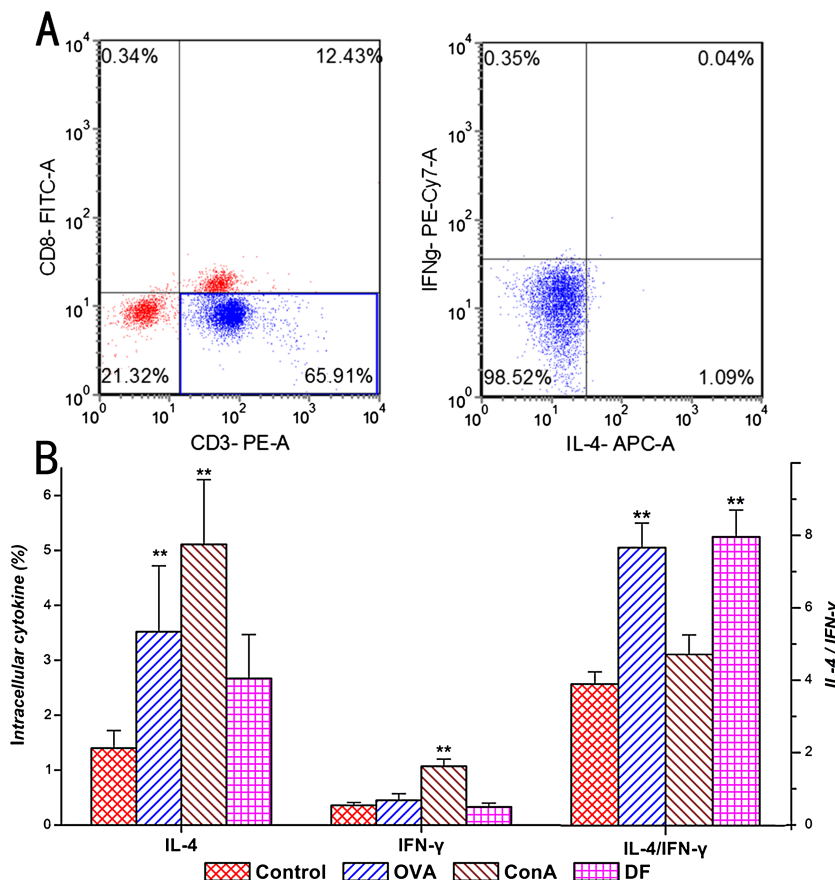


Figure 4. Detection of intracellular cytokines stained in single cell of PLN. As showed in representative scatter-plots (A), CD3⁺CD8⁻ gating for CD4⁺ T cells intracellular IL-4 and IFN- γ analysis in cells isolated from PLNs by flow cytometry are presented. The intracellular IL-4 and IFN- γ percentages was showed in Fig 4B quantified on the left-axis, and the ratios of intracellular IL-4 and IFN- γ are also presented in Fig 4B quantified on the right-axis. Results are expressed as mean $\pm$ SEM with 4-6 mice in each group. Significant differences are indicated by * $p < 0.05$ and ** $p < 0.01$ compared with control.

significantly over the past 10 years and has been garnering greater concern in recent years (Fei *et al.*, 2009). Therefore, an intravenous exposure model is urgently needed for identification of IDHR potential induced by TCMI. However, unfortunately, to the best of our knowledge, there is still a lack of a valid model for drug-induced hypersensitivity assessments in intravenously administered drugs to date.

RA-PLNA for screening of immunosensitizing chemicals and their immunosensitizing mechanisms have been reported previously in IDHR identification models (Nierkens *et al.*, 2002; Pieters and Albers, 1999). However, injection into the footpad is not a normal route of exposure, and may cause more unexpected deviations in the results than more frequently used clinical routes of drug administration (Goebel *et al.*, 1996). Thus, the development of models of assessment for such drug exposure in humans is necessary. The development of an oral murine food allergy model for peanut allergens (van Wijk *et al.*, 2004) and an oral exposure mouse model for drug-induced allergy prediction (Nierkens *et al.*, 2005b) has been reported. However, to the best of our knowledge, no intravenous exposure model has been reported to date. Therefore, in this study, we introduced an intravenous drug-administration mouse model that seems to be useful for identification of IDHR caused by drugs using RA-PLNA.

Owing to the complex constituents of TCMI, including proteins and LMWCs, two protein antigens (OVA and ConA) and an LMWC (DF) were selected as positive compounds in this study. OVA, inducing IgG-mediated systemic anaphylaxis through both IgE- and IgG-mediated mechanisms, is used as a sensitizer in anaphylaxis mouse models that are administered orally (Yoshida *et al.*, 2011). ConA acts on T cell proliferation via a totally different mechanism and enhances the mixture reaction in mice (Doyle *et al.*, 1978; Oliver *et al.*, 1996). Diclofenac, a nonsteroidal anti-inflammatory drug, is known to be able to enhance sensitization (de Groot *et al.*, 2009; van Puijenbroek *et al.*, 2002); it is used as a referent positive compound in LMWC and induces anaphylaxis assessment for potential prediction in mouse models (Gutting *et al.*, 1999, 2002a, b). Therefore, OVA, ConA and DF were selected as positive compounds to validate the model in this study.

In the study, our results showed that, by using the RA-PLNA, all positive compounds could be clearly identified as provoking hypersensitivity on intravenous injection exposure in mice. No reactions were promoted by the negative compound. Thus, based on these results, the intravenous drug-exposure murine model appears to be effective for identifying immunosensitizing drugs that are administered intravenously.

Cytokine fingerprinting has been found to be helpful in identifying a reaction owing to drug-induced hypersensitivity (Dearman *et al.*, 2009a; Lovik *et al.*, 2007). Obviously, in terms of lymph node parameters, weight and cellularity are associated with irritant and immune responses (allergic and/or nonallergic). Therefore, specific antibodies associated with allergy measurements (or enumeration of antibody-producing cells) are necessary to distinguish whether a lymph node response is relevant with regard to allergy. The RA-specific AFCs induced with the compounds provided information concerning the type of immune responses elicited by drugs. Collectively, detection of RA-specific AFCs can clearly improve the sensitivity and selectivity of the PLNA. Furthermore, PLN data could be adequate for screening purposes and providing mechanistic information on drug-induced hypersensitivity.

In addition, differentiation of immune responses may also be analyzed by RA-specific AFC isotypes associated with Th1 and Th2. In our results for the present study, intravenous injection of OVA (20 and 40 mg kg⁻¹) significantly increased numbers of TNP-specific IgG1, without increase in IgG2a and IgG2b AFCs, suggesting induced Th1-type immune response. Low doses of ConA, however, were shown to induce the Th1 response (with IgG1 and IgM increase), and high doses (10 and 20 mg kg⁻¹) seemed to induce a more mixed Th1/Th2 immune response (with IgG1, IgE, IgG2a and IgG2b increase). A recent study on DF demonstrated that it does not undergo metabolism in viable human skin (Tanojo *et al.*, 1999), therefore, an intravenous exposure model might be of value in identification of metabolic activators. Data on TNP-specific antibodies from DF showed that intravenous administration of DF (10 and 40 mg kg⁻¹) significantly induced Th1 immune response.

Previously reported studies have suggested that TNP-OVA PLNA had an advantage in providing information on the role of the T-cell response, a major limitation in direct PLNA (Albers *et al.*, 1997; Goebel *et al.*, 1996). It has been reported that most test compounds induce PLN responses following activation of CD⁴⁺ T cells, divided into T-helper 1 and T-helper 2 cells, by MHC II molecules (Descotes, 2000). Anaphylaxis is classified as a Th2 response, which is mediated by IgE and/or IgG1 synthesis that depends on interleukin 4 (IL-4), in contrast to Th1 responses to IFN- γ that depends on IgG2a or IgG2b production (Kerdine *et al.*, 1996). Although TNP-specific antibody AFCs may analyze T-cell activations, they provide this evidence indirectly. Therefore, these cells may contribute to the effects observed in this study, as well as the results of PLN weight indexes and cell numbers, such as the significant increase in CD¹⁹⁺ subpopulation cells (B cells) in PLNs treated with each positive compound. Although OVA and DF did not manifest significant increases in CD³⁺ T cells, numbers of CD⁴⁺ cells were enhanced, and significant differences were manifested by OVA as compared with the control. Corresponding with the intravenous cytokine results, OVA significantly increased the percentage of intravenous IL-4⁺ cells, and both OVA and DF enhanced the ratio of IL-4/IFN- γ . For ConA, all CD³⁺, CD⁴⁺ and CD⁸⁺ cell numbers were significantly increased, as well as the percentage of IL-4⁺ and IFN- γ ⁺ cells. Collectively, by intravenous exposure, OVA induced CD⁴⁺ Th2-cell-dependence Th2 responses with IgG1 AFCs increased, and similarly for DF. In addition ConA induced a more mixed response.

In conclusion, by using RA-PLNA, the intravenous drug-exposure mouse model presented here appears to be a potentially useful tool for assessing drug-induced immunosensitizing hazards screened via intravenous exposure. With the use of PLN cell read-out parameters, the model maybe a suitable tool for screening a range of different drugs in routine IDHR assessments preclinically, and may at least warrant more careful and extensive assessment in the clinical phase, especially for TCMI.

Conflict of Interest

The authors have no potential conflicts of interest.

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