ARTIGO
https://doi.org/10.22239/2317-269x.01088

Local lymph node assay in the differentiation between allergic and irritant contact dermatitides: study of the expression of T lymphocyte surface markers

Ensaio do linfonodo local murino (LLNA) na diferenciação entre a dermatite de contato alérgica e de contato irritante: um estudo da expressão de marcadores de superfície de linfócitos T

Bárbara Andréa Fortes dos Santos[I,*]

Edson Roberto Alves de Oliveira[II]

Marciano Viana Paes[III]

Fabio Coelho Amendoeira[IV]

Isabella Fernandes Delgado[V]

Ana Cristina Martins de Almeida Nogueira[III]

[I] Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brasil

[II] Instituto de Química, Centro de Tecnologia, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brasil

[III] Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (IOC/Fiocruz), Rio de Janeiro, RJ, Brasil

[IV] Instituto Nacional de Controle de Qualidade em Saúde, Fundação Oswaldo Cruz (INCQS/Fiocruz), Rio de Janeiro, RJ, Brasil

[V] Vice-Presidência de Educação, Informação e Comunicação, Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, RJ, Brasil

* **E-mail:** barbarafortes@hotmail.com

Received: Nov 22, 2017
Approved: Aug 08, 2018

ABSTRACT

Introduction: The Local Lymph Node Assay (LLNA) was developed as an alternative to the Buehler and maximization tests. It is applied to discriminate substances that are able to induce contact dermatitis and its outcome is cell quantification in mice auricular lymph nodes. Although recommended by international agencies involved in the development of alternative methodologies, the LLNA still needs to be improved. **Objective:** To investigate possible differences in lymphocyte subpopulation patterns between mice treated with irritants and skin sensitizing agents. **Method:** Animals were treated with the sensitizing substances dinitrochlorobenzene and paraphenylenediamine and the irritants sodium lauryl sulfate and Triton X-100 for three days by applying the substances on the back of both ears. The percentages of different lymphocyte subpopulations were analyzed using flow cytometry. The ears of the animals were also evaluated for possible pathological alterations. **Results:** Differences were observed in CD4+CD25+ and CD4+CD69+ cells, as well as in the proliferation of these subpopulations. Histopathological analysis of the ears showed no difference between the treatments with skin sensitizing agents and irritants. **Conclusions:** T lymphocyte phenotyping may be useful for developing an assay to differentiate between skin sensitizing agents and irritants. Additionally, these results may contribute to improving the knowledge of this field and help in the search for a correlate *in vitro* assay.

KEYWORDS: Contact Dermatitis; LLNA; Alternative Method; Health Surveillance

RESUMO

Introdução: O ensaio do linfonodo local murino (LLNA) foi desenvolvido como uma alternativa aos testes de Buehler e maximização. O teste tem o objetivo de identificar substâncias capazes de induzir dermatite de contato e tem como desfecho a quantificação celular nos linfonodos auriculares. Embora recomendado por agências internacionais envolvidas no desenvolvimento de metodologias alternativas, o LLNA ainda necessita de aprimoramento. **Objetivo:** Estudar possíveis diferenças nos padrões de subpopulações linfocitárias entre camundongos tratados com substâncias irritantes e dermosensibilizantes. **Método:** Os animais foram tratados com os sensibilizantes dinitroclorobenzeno e parafenilenidiamina e os irritantes lauril sulfato de sódio e Triton X-100 por três dias consecutivos no dorso de ambas as orelhas. As subpopulações foram analisadas por citometria de fluxo e possíveis alterações histopatológicas nas orelhas dos animais foram examinadas. **Resultados:** Foram observadas diferenças nas células CD4+CD25+ e CD4+CD69+, assim como na proliferação dessas subpopulações. Nenhuma diferença entre os tratamentos com dermosensibilizantes e irritantes foi vista nos estudos histopatológicos das orelhas dos animais. **Conclusões:** A fenotipagem de linfócitos T pode ser considerada útil no desenvolvimento de possíveis protocolos de ensaios que visem à diferenciação entre substâncias dermosensibilizantes e irritantes. Além disso, os resultados obtidos podem vir a contribuir com o aumento do conhecimento nesta área e auxiliar na busca por um ensaio *in vitro* correlato.

PALAVRAS-CHAVE: Dermatite de Contato; LLNA; Método Alternativo; Vigilância Sanitária

INTRODUCTION

Occupational skin diseases are a public health concern. The number of products incorporated into the environment grows annually, thus increasing the potential of these toxic agents to induce adverse events. In addition, in 2012, the annual direct and indirect costs related to occupational skin diseases exceeded US$ 1 billion in the United States only. Expenses with dermatological treatments are expected to reach US$ 18.5 billion per year until 2018[1].

The development of methodologies aimed to predict and control possible adverse effects of xenobiotics on the immune system is a pertinent subject and a relevant challenge for health surveillance. Hypersensitivity reactions are the most prevalent adverse effects caused by xenobiotics[2,3,4,5]. Contact dermatitis (CD) is an inflammatory dermatosis induced by chemical agents. It encompasses mainly allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD)[6]. The former is a type of CD provoked by repeated contacts between the skin and small reactive molecules with molecular weight lower than 500 Da called haptens, originating from several sorts of chemical agents, which are recognized by the immune system as foreign antigens[7].

Allergic contact dermatitis requires the activation of innate and adaptive immunity and, according to several authors, is mediated mostly by T cells[6,8]. Genetic factors also play a role in the development of this disease[9]. Contact dermatitis is set off by the topical application of sensitizing agents on the epidermis, such as nickel, chromium, dinitrofluorobenzene (DNFB), and oxazoline (OXA)[10,11]. Consequently, the immunopathological mechanism of ACD begins at the first contact between the skin and the sensitizing agent, which results in the formation of a hapten-protein conjugate that can be recognized and captured by Langerhans cells. When this process occurs, these cells can move from the epidermis to the paracortical region of drainage lymph nodes. The presentation of these peptides associated with haptens activates specific CD8 and CD4 T lymphocytes[12,13,14]. Reexposure of the same hapten results in the production of proinflammatory cytokines and chemokines by epidermal cells, recruitment of memory T lymphocytes, activation of endothelial and mast cells, and infiltration of neutrophils. All these phenomena are necessary for this inflammatory process to develop[12,15,16]. The final step of the immunological mechanism of ACD is the end of the inflammatory reaction. At this phase cytokines are released, including IL-10, which, conversely to those in the previous phases, seems to be more related to the inhibition of the immunological reaction[14,17].

Irritant contact dermatitis, for its turn, is characterized by a skin inflammation that develops after immediate contact with irritant substances[18]. The immunological response described for this type of dermatitis involves innate immunity, more specifically an increased production of proinflammatory cytokines in cells that make up the epidermis and dermis, such as keratinocytes and Langerhans cells, which secrete several cytokines, among which the tumor necrosis factor-alpha (TNF-a), granulocyte-macrophage colony-stimulating factor, and IL-1[13,19].

However, there are controversies regarding the participation of the adaptive immunological system in ICD. It is known that some substances classified as irritants may induce the proliferation of adaptive response cells[20,21,22].

For a long time, ICD was defined as a nonspecific reaction of the skin to the presence of irritants. At present, it is accepted that the immune system plays a fundamental role in triggering ICD. In the presence of irritant agents, physiopathological changes may take place, such as destruction of the epithelial barrier, cell damage, and increase in the level of proinflammatory mediators[18,23,24]. A cell type that is crucial in the immunological response set off by irritants is keratinocytes. They give off cytokines during the destruction of the epithelial barrier, express class II histocompatibility molecules, adhesion molecules (CD54), and costimulatory molecules, such as CD80 and CD40[25]. The release of cytokines by keratinocytes stimulates other keratinocytes and cell types, such as Langerhans cells, and attracts inflammatory cells, thus enhancing the inflammatory process[26]. Proinflammatory cytokines, such as IL-1a, IL-1b, TNF-a [27], IL-6, IL-2[26], and the CCL21 chemokine, produced by lymphatic endothelial cells, were found at higher levels in people with ICD[28,29].

The investigation of subpopulations of lymphocytes involved in ACD and ICD has proved promising. The possibility of using lymphocyte phenotyping as a method to distinguish between ACD and ICD has been examined. Studies of subpopulations of lymphocytes show several proposed guidelines and few similarities, which hinders the elaboration of a common protocol. Some authors demonstrated an increase in B cells, measured via the presence of B220 and CD19 receptors in mice exposed to skin sensitizing agents[30,31]. Lee et al.[32] partially confirmed the results of the previous investigations, because they observed a selective increase in the percentage of B cells that presented the CD86 marker only when exposed to sensitizing agents, but did not obtain conclusive results about the CD40 molecule[32]. Neves et al.[33] reported a growth in the level of the CXCR4 chemokine receptor and CD40 molecule *in vitro* in dendritic cells when these were treated with sensitizing substances. Nevertheless, when these cells were exposed to irritants, the expression of these molecules was downregulated[33].

Additionally, there are several controversies regarding the study of subpopulations of T lymphocytes. There is no consensus on either the subpopulations of CD4 T cells (auxiliary T cells) or CD8 T cells (cytotoxic T cells). Goutet et al. observed and analyzed the differences in the expression of the IL-4 cytokine and its receptor in CD4 T cells[34], whereas Lee et al. investigated the percentages of CD4 and CD8 T cells only[32]. De Silva et al. studied the population of total T cells by using CD3 and CD25, which is the IL-2 cytokine receptor and, together with CD4, is the marker of regulatory T cells[35]. Furthermore, *in vitro* studies with dendritic cells originating from the bone marrow (BMDC) proved promising to assess the allergic potential of chemical products. Battais et al. showed that the expression of molecules such as

MHC II, CD86, CD54, and CD40 in BMDC may distinguish between allergens and nonallergens and classify chemical substances according to their allergenic potential[36]. However, it is important to stress that the field literature has few papers addressing this subject. Consequently, studies of this type are still necessary to reproduce the data reported in previous investigations and increase the fundamental knowledge of the topic, aiming to reach a consensus.

Another relevant aspect in this type of study is the use of laboratory animals. Although Asherson and Barnes[37] used mice as a model to predict hypersensitivity reaction, other investigations were carried out with guinea pigs, and consequently the first tests aimed to predict hypersensitivity reactions induced by xenobiotics were developed using this animal. Over the years, several experiments were proposed and their applicability and limitations have been discussed up to the present[38,39,40,41,42].

The Buehler[43] and maximization[44] tests were designed to identify the potential of substances to cause ACD[43,44]. Although widely used in the 1970s and 1980s, these procedures had serious limitations in common, such as: (i) subjective data interpretation (evaluation of the extension of the local edema, for instance); (ii) long duration of the tests (both executed in two phases, with a total period between 25 and 30 days); (iii) high costs and a significant demand for labor; and (iv) use of a high number of animals, which were submitted to a considerable level of stress[38,45]. At the end of the 1980s, the local lymph node assay (LLNA) emerged as a promising alternative to the conventional tests carried out with guinea pigs.

The local lymph node assay was first described by Kimber and Weisenberger[46] as a substitute for the Buehler and maximization tests[47,48,49,50] and was accepted for regulatory purposes in 2002[51] by the *European Centre for the Validation of Alternative Methods* and the *Interagency Coordinating Committee on the Validation of Alternative Methods*. The main advantages of this test in comparison with those of its precursors are: (i) the analyzed final parameter (proliferation of lymphocytes assessed through ^{3}H-thymidine) is quantitative; (ii) the test is performed during the sensitization phase, and consequently is short (five days); (iii) characteristics of the examined substance, such as color, do not influence the test outcomes; (iv) the number of animals used per substance-test is around 50% lower than that used in the other tests involving guinea pigs; and (v) it spares animals pain and suffering resulting from phase 2 reactions, which is the inflammatory phase[46,52].

However, the LLNA protocol initially accepted for regulatory purposes[51] also presented important limitations, such as the use of radioactive material and delivery of false positives[22,53,54,55,56]. Because of this, as part of the improvement of the assay, modified LLNA protocols were launched in 2010[57,58]. Additionally, the definitive prohibition of the use of animals by the cosmetics industry in Europe in 2013[59] intensified the search for *in vitro* methodologies. In this context, predictive strategies, such as the *Integrated Approaches to Testing and Assessment, have stood out worldwide and drawn the attention of regulatory agencies*[60]. *Many in vitro assays that do not have a significant predictive capacity in isolation become promising strategies when combined in a battery of tests. Thus, many strategies and batteries of assays proposed by the scientific community have been studied and assessed systematically by centers of validation of alternative methods, and some have already gained regulatory acceptance, including in the skin sensitization field*[57,58,60,61,62,63].

Regulatory agencies and the scientific community have sought advances in reaching consensuses, and one of them regards the use of new approaches to cope with the challenges of 21st century toxicology. Many authors believe that regulatory toxicology must resort to modern and innovative approaches, such as the use of big data and *in silico*, "omics", and high-throughput models, among others, and deepen mechanistic studies to better understand the toxicological outcomes that are aimed to be predicted[64,65,66]. The present study was designed in this context, and had the objectives to examine the mechanism of differentiation between ACD and ICD by using lymphocyte phenotyping and increase the knowledge of the physiopathological alterations involved in the execution of the LLNA test.

METHODS

Animals

All the procedures involving animals were approved by the Animal Research Ethics Committee as per license no. P-0415/07 and carried out in accordance with the *Animal Research: Reporting of In Vivo Experiments protocol*. *Treatment groups had four animals for each tested substance and another four control animals treated with the carrier only. Female mice from the CBA strain with ages ranging from eight to ten weeks and an average weight of 17 g were used. The animals were provided by the Science and Technology Institute for Biomodels* and kept at the Animal Experimentation Division of the National Institute of Health Control of the Oswaldo Cruz Foundation in cages with the dimensions 20 cm x 35 cm x 15 cm. The animals had free access to water and food and were submitted to diurnal and nocturnal cycles of 12 hours and controlled temperature (20 ºC to 26 ºC) and humidity (30% to 70%). Two animals from each cage were randomly selected to be treated with the substances and carriers. The treatment was carried out during the diurnal cycle in a laminar flow cabinet. A volume of 25 µL of each tested substance or carrier was applied with a tip to the back of both ears of the animals for three consecutive days. Two hours before euthanasia by CO_2 inhalation, the animals received 100 mg/kg of bromo-2'-deoxyuridine (BrdU) in 200 µL of salt solution, in accordance with the LLNA protocol[51].

Chemicals

The substances used in the experiment were as follows: severe (paraphenylenediamine (PPD, concentration of 1%) and

dinitrochlorobenzene (DNCB, concentration of 1%) diluted with acetone (A)/olive oil (OO) (A:OO 4:1)) and moderate skin sensitizing agents (sodium lauryl sulfate (SLS, concentration of 25%) and Triton® X-100 (TX-100, concentration of 25%) diluted with 30% ethanol. All the chemicals were purchased from Sigma-Aldrich (USA). The reagent BrdU was obtained from the same company. The antibodies used were: anti-CD4 allophycocyanin, anti-CD69 phycoerythrin (PE), anti-CD25 PE and fluorescein isothiocyanate (FITC), anti-CD11a PE, and anti-BrdU FITC. All of them were purchased from BD Pharmingen, USA.

LLNA and flow cytometry

After euthanasia, ear lymph nodes were isolated and macerated using a tissue homogenizer to obtain a cell suspension, which was then centrifuged for 5 minutes at 300 g and ressuspended in 1% PBS/BSA. Subsequently, cells were counted using a Neubauer chamber and their concentration was adjusted to $1x10^6$ cells/mL. They were incubated for 30 minutes with the antibodies listed previously. For the labeling with BrdU, cells were permeabilized in a solution of 70% ethanol at 20 °C for 30 minutes. The cell suspension was centrifuged twice for 5 minutes at 300 g and incubated with 100 µL of deoxyribonuclease for 40 minutes at 25 °C. Cells were then labeled with anti-BrdU for 20 minutes at 4 °C. The resulting samples were analyzed using flow cytometry (FACSCalibur, Becton, Dickinson and Co., Franklin Lakes, NJ, USA). The results were treated using the WinMDI version 2.9 software.

Histological analysis

The ears of the animals were removed and fixed in a Millonig solution for 24 hours. The material was then dehydrated in ethanol, diaphanized in xylol and incorporated into paraffin[67]. The paraffin blocks containing the ears were cut to have a dimension of 5 µm and laid on microscope glass slides. The samples were stained with hematoxylin and eosin (H&E) and analyzed using a Nikon Eclipse E600 microscope.

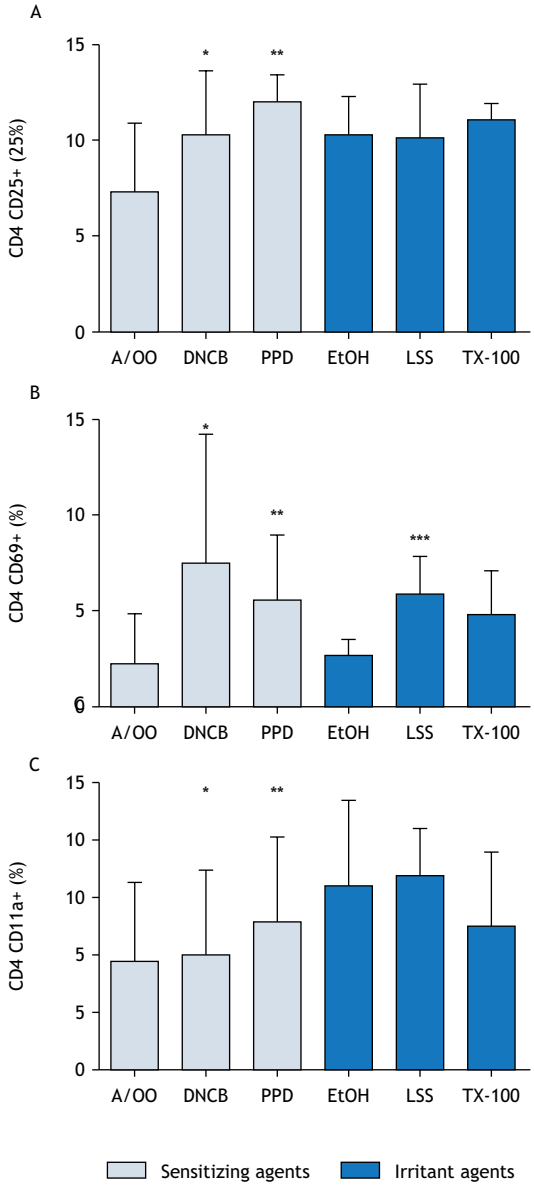
Statistical analysis

The nonparametric Mann-Whitney test was run with the Graphprism 5.1 program. Data were considered statistically significant when the p value was lower than 0.05.

RESULTS

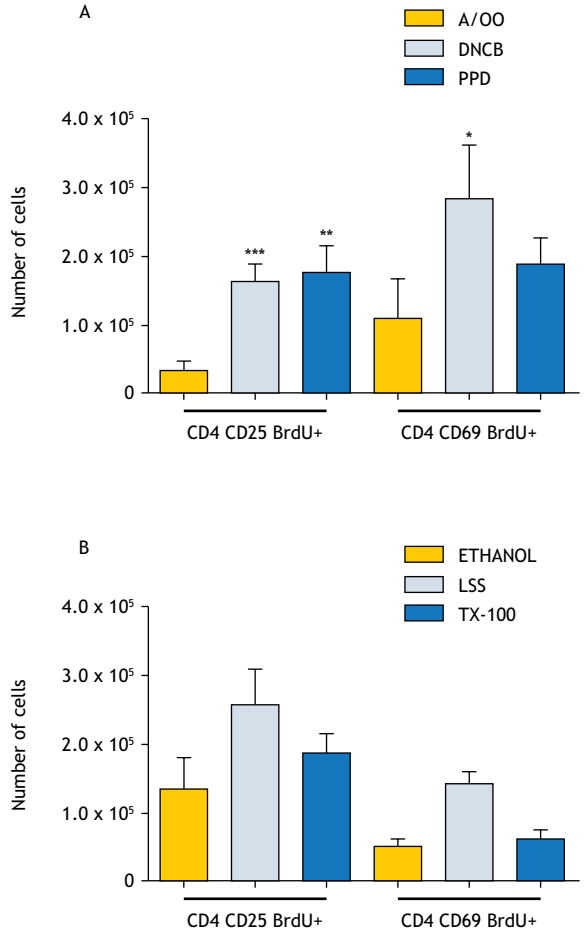
Possible T lymphocyte markers for differentiation between ACD and ICD

The expressions of some cell activation markers, such as the a chain of the IL-2 (CD25) receptor and the CD69 receptor, and of the adhesion marker CD11a, which can enhance its expression intensity when faced with cell activation, proved possible candidates for differentiation between ACD and ICD (Figure 1). A significant increase of CD4+CD25+ cells was observed in animals treated with the skin sensitizing substances DNCB and PPD. This growth was also found for CD4+CD69+ cells when the animals were exposed to PPD. However, when animals were submitted to contact with the irritant substances, the increase in CD4+ cells was significant for the CD69 activation marker only, and this increase occurred exclusively in animals treated with SLS in comparison with the control substance ethanol. No significant difference was found in the percentage



(A) Percentage of CD4+CD25+ cells in animals treated with the skin sensitizing substances DNCB and PPD and their vehicle A/OO (control) and irritants SLS and TX-100 and their carrier ethanol (control). (B) Percentage of CD4+CD69+ cells treated with the skin sensitizing substances DNCB and PPD and their carrier A/OO (control) and irritants SLS and TX-100 and their vehicle ethanol (control). (C) Percentage of CD4+CD11a+ cells treated with the skin sensitizing substances DNCB and PPD and their vehicle A/OO (control) and irritants SLS and TX-100 and their vehicle ethanol (control). Bars represent the average + standard deviation for three independent experiments, with four animals in each group.
*p < 0.050; **p < 0.010; ***p < 0.001 show that there was a statistically significant difference in comparison with the control group according to the nonparametric Mann-Whitney test with a confidence interval of 95%.

Figure 1. Expression of activation and adhesion markers.



(A) Significant increase of CD4+CD25+BrdU+ and CD4+CD69+BrdU+ subpopulations in animals treated with DNCB and PPD in comparison with the result obtained in the control group, treated with A/OO. (B) Nonsignificant increase of CD4+CD25+BrdU+ and CD4+CD69+BrdU+ populations in animals treated with SLS and TX-100 in comparison with the result obtained in the control group, treated with ethanol. Bars represent the average + standard deviation for three independent experiments, with four animals in each group.
*p < 0.050; **p < 0.010; ***p < 0.001 show that there was a statistically significant difference in comparison with the control group according to the nonparametric Mann-Whitney test with a confidence interval of 95%.

Figure 2. Proliferative increase of subpopulations.

of CD4+CD25+ cells. Neither the treatment with irritant substances nor with skin sensitizing ones induced significant alterations in the adhesion marker CD11a.

Although an effect on T cell subpopulations was observed, it was necessary to confirm the proliferation of these cells, given that the outcome of LLNA is cell proliferation (Figure 2). Consequently, the next step was to evaluate whether the effect observed in T cells would be confirmed with a proliferation profile using BrdU staining. The absolute number of cells in CD4/CD25/BrdU+ and CD4/CD69/BrdU+ subpopulations was analyzed in the different treatment groups. Some significant differences were found. The group of animals exposed to DNCB showed an increase in the CD4+CD69+BrdU+ subpopulation, whereas a growth in the CD4+CD25+BrdU+ subpopulation was registered in the animals treated with DNCB and PPD in comparison with data related to mice treated with the A/OO carrier. When the animals were exposed to irritants, no significant differences were observed between the groups treated with SLS and TX-100 in comparison with the results obtained in the group treated with ethanol.

Histological alterations in the ears of mice

Several studies showed that the thickness of the ears of mice increases considerably after treatment with skin sensitizing substances and irritants for three consecutive days in comparison with the result observed in control animals[12,13,68,69,70]. This outcome has been suggested in the field literature as an improvement of the LLNA. Nevertheless, comparative histopathology data of the ears of animals are limited. The present investigation included a preliminary histopathological examination of the ears of the animals treated with skin sensitizing and irritant substances.

No pathological tissue alterations were found in the groups treated with the vehicle ethanol and A/OO, as revealed by histological examination (Figure 3A and 3D). Both sensitizing (DNCB and PPD) (Figure 3B and 3C) and irritant (SLS and TX-100) substances induced epidermal hyperplasia and cellular infiltrate in the ears of the animals, as shown in Figures 3E and 3F.

DISCUSSION

One of the first cell surface antigens expressed by T cells after activation is CD69. Once expressed in cells, it functions as a costimulatory molecule in T cell proliferation[71]. CD25 is the a chain of the IL-2 receptor and is expressed in activated T and B cells. In the present study, analysis of T lymphocytes regarding the activation markers CD25 and CD69 revealed an increase in the absolute number of CD4 cells expressing these two activation markers. This growth in the number of CD4+CD25+ cells occurred in the lymph nodes of animals treated with the skin sensitizing agents DNCB and PPD but not in the group exposed to irritants. For the CD4+CD69+ cell subpopulation, this increase was registered in animals treated with PPD and SLS. The results obtained in the experiment with the sensitizing substances corroborate the findings of Homey et al.[72]. These authors reported that topical treatment with the skin sensitizing agent OXA caused an increase in the number of cells expressing the activation markers CD25 and CD69 in comparison with data obtained for the control group, and it was higher in CD4+ cells than in CD8+ cells. The authors also found that there was no significant increase after exposure to an irritant substance (croton oil) in comparison with the results in the control group[72]. In a more recent study, Strauss et al.[73] examined the immunophenotyping of lymphocytes in the LLNA by comparing several sensitizing and irritant substances. The authors monitored pan surface markers for B, TCD4, and TCD8 cells. In addition, they measured the expression of CD69 and MHC-II (I-A^k) in lymphocyte subpopulations. The authors found no differences in the number of cells presenting these profiles when they compared results from animals treated with sensitizing agents with those

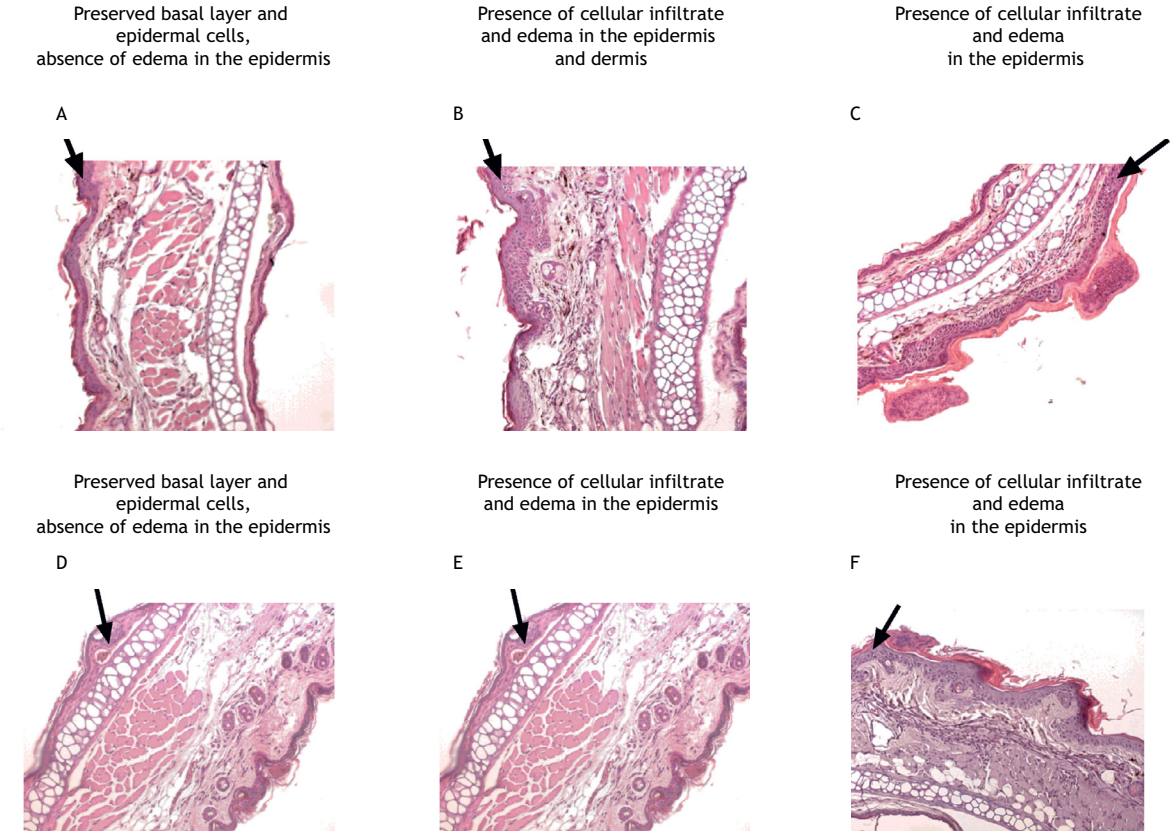


Figure 3. Alteration in the ears of the animals. Hematoxilin and eosin (H&E)-stained images of the ears of animals treated with: the vehicles (A) A/OO and (D) ethanol, revealing the absence of inflammatory infiltrate and edema in the epidermis; (B) DNCB and (E) SLS, showing the presence of inflammatory infiltrate and edema in the epidermis; (C) PPD and (F) TX-100, revealing the presence of inflammatory infiltrate and edema in the epidermis. 20X magnification.

from animals exposed to irritants. These findings match those obtained in the present study. However, taking into account the significant difference in the CD4+CD25+ subpopulation, which was not mentioned in the paper by Strauss et al., there is a high possibility of finding a secure outcome in immunophenotyping[73]. Furthermore, another study[74], which used the popliteal lymph node assay (also applied in the evaluation of immunosensitizing agents), indicated the CD25 molecule, among others, as a possible marker for the determination of activation of T cells by immunosensitizing substances.

Regarding the BrdU marker in these subpopulations, there were no significant changes in the lymph nodes of animals treated with SLS and TX-100, but the results indicate an increase in the number of CD4+CD69+BrdU+ and CD4+CD25+BrdU+ cells in animals treated with the substance SLS in comparison with those exposed to ethanol. Although most irritants lead to negative results in this type of test, studies show that some irritant substances induce cellular accumulation and proliferation, just like sensitizing agents[2,55,75,76]. Jung et al. reported an increased incorporation of BrdU after treatment with SLS[75]. However, concerning animals treated with sensitizing molecules, the present study revealed an increased quantity of BrdU+ in CD4+CD69+ cells in animals exposed to DNCB and in CD4+CD25+ cells in animals treated with DNCB and PPD.

Additionally, possible histological alterations in the ears of the animals were analyzed to be considered for the differentiation between ACD and ICD. Data collected in the present study do not show differences between the treatment with irritant and sensitizing substances. Lee et al.[77] observed a proliferative increase using BrdU in epidermal cells in animals treated with DNCB, toluene diisocyanate, hexyl cinnamic aldehyde, and SLS in comparison with their controls. Dubois et al.[78] analyzed the contribution of TCD4 and TCD8 cells to primary ACD, which occurs when people develop a CD after a single application of a strong sensitizing substance. Using monoclonal antibodies, the animals had their TCD4 and TCD8 cells depleted and were treated with the severe skin sensitizing agent DNFB. The animals which had their TCD4 cells depleted presented a larger edema in the dermis and significant cellular infiltration in comparison with normal animals treated with DNFB. Conversely, animals without TCD8 cells showed no pathological alteration.

Bonneville et al.[13] showed that the ACD response is more severe in mice from the C57Bl/6 strain than in those from the BALB/c strain. Mice from both strains were treated with DNFB and the thickness of their ears was evaluated. Three hours after the exposure, C57BL/6 mice had an edema larger than that observed in BALB/c mice. Similarly, histological analysis demonstrated that six hours after the treatment C57Bl/6 mice had a cellular

infiltrate in the dermis and a edema larger than those present in BALB/c mice[13]. Jung et al. reported an increase in the weight of the ears treated with 1% SLS and PPD in comparison with control animals and analysis of the histological aspect revealed epidermal hyperplasia and inflammatory infiltrate[75]. Similarly, Ku et al.[70] showed that the skin sensitizing agents DNCB and OXA and the irritant croton oil induce epidermal hyperplasia and inflammatory infiltrate. The findings of the present study corroborate the results described by Jung et al.[75] and Bonneville et al.[13], given that the same histological alterations, that is, hyperplasia and infiltrate, were observed after treatment with skin sensitizing agents and irritants.

CONCLUSIONS

The results show differences in both phenotyping and proliferation of T lymphocytes in animals treated with the skin sensitizing agents DNCB and PPD in comparison with the results obtained in the control group, treated with A/OO, and the group treated with SLS and TX-100. These data are relevant and may be useful for developing predictive strategies and assay protocols to distinguish between ACD and ICD. Histopathological analysis of animals that received topical treatment with skin sensitizing agents and irritants does not seem to significantly help distinguish between these two categories of substances.

REFERENCES

1. Wong CC, Gamboni SE, Palmer AM, Nixon RL. Occupational allergic contact dermatitis to chromium from cement: estimating the size of the problem in Australia. Australas J Dermatol. 2015;56(4):290-3. https://doi.org/10.1111/ajd.12238
2. Basketter DA, Kimber I. Skin irritation, false positives and the local lymph node assay: a guideline issue? Regul Toxicol Pharmacol. 2011;61(1):137-40. https://doi.org/10.1016/j.yrtph.2011.07.004
3. Lidén C, Yazar K, Johansen JD, Karlberg AT, Uter W, White IR. Comparative sensitizing potencies of fragrances, preservatives, and hair dyes. Contact Dermat. 2016;75(5):265-75. https://doi.org/10.1111/cod.12683
4. Chen W, Xing C, Hou F. Intra-laboratory study to determine the reproducibility of LLNA:BrdU-ELISA for the prediction of the skin sensitizing potential of chemicals. J Pharmacol Toxicol Methods. 2016;82(82):26-30. https://doi.org/10.1016/j.vascn.2016.07.003
5. Lee YS, Yi JS, Seo SJ, Kim JH, Jung MS, Seo IK et al. Comparison of BALB/c and CBA/J mice for the local lymph node assay using bromodeoxyuridine with flow cytometry (LLNA: BrdU-FCM). Regul Toxicol Pharmacol. 2017;83:13-22. https://doi.org/10.1016/j.yrtph.2016.10.021
6. Shin EJ, Gwak MJ, Jeong KH, Shin MK. Lack of differentiation of allergic and irritation reactions by skin autofluorescence. Contact Dermat. 2017;76(5):318-21. https://doi.org/10.1111/cod.12708
7. Balmert SC, Donahue C, Vu JR, Erdos G, Falo LD Jr, Little SR. In vivo induction of regulatory T cells promotes allergen tolerance and suppresses allergic contact dermatitis. J Control Release. 2017;261:223-33. https://doi.org/10.1016/j.jconrel.2017.07.006
8. Martins LE, Reis VM. Immunopathology of allergic contact dermatitis. An Bras Dermatol. 2011;86(3):419-33. https://doi.org/10.1590/S0365-05962011000300001
9. Schnuch A, Westphal G, Mössner R, Uter W, Reich K. Genetic factors in contact allergy: review and future goals. Contact Dermat. 2011;64(1):2-23. https://doi.org/10.1111/j.1600-0536.2010.01800.x
10. Peiser M, Tralau T, Heidler J, Api AM, Arts JH, Basketter DA et al. Allergic contact dermatitis: epidemiology, molecular mechanisms, in vitro methods and regulatory aspects. Current knowledge assembled at an international workshop at BfR, Germany. Cell Mol Life Sci. 2012;69(5):763-81. https://doi.org/10.1007/s00018-011-0846-8
11. Pongpairoj K, Puangpet P, Thaiwat S, McFadden JP. Diagnosing allergic contact dermatitis through elimination, perception, detection and deduction. Am J Clin Dermatol. 2017;18(5):651-61. https://doi.org/10.1007/s40257-017-0287-8
12. Hennino A, Saint-Mezard P, Nicolas J, Vocanson M, Dubois B, Chavagnac C et al. Update on the pathophysiology with special emphasis on CD8 effector T cells and CD4 regulatory T cells. An Bras Dermatol. 2005;80(4):335-47. https://doi.org/10.1590/S0365-05962005000400003
13. Bonneville M, Chavagnac C, Vocanson M, Rozieres A, Benetiere J, Pernet I et al. Skin contact irritation conditions the development and severity of allergic contact dermatitis. J Invest Dermatol. 2007;127(6):1430-5. https://doi.org/10.1038/sj.jid.5700726
14. Bonefeld CM, Geisler C, Gimenéz-Arnau E, Lepoittevin JP, Uter W, Johansen JD. Immunological, chemical and clinical aspects of exposure to mixtures of contact allergens. Contact Dermat. 2017;77(3):133-42. https://doi.org/10.1111/cod.12847
15. Nosbaum A, Vocanson M, Rozieres A, Hennino A, Nicolas JF. Allergic and irritant contact dermatitis. Eur J Dermatol. 2009;19(4):325-32. https://doi.org/10.1684/ejd.2009.0686
16. Toebak MJ, Gibbs S, Bruynzeel DP, Scheper RJ, Rustemeyer T. Dendritic cells: biology of the skin. Contact Dermat. 2009;60(1):2-20. https://doi.org/10.1111/j.1600-0536.2008.01443.x
17. Honda T, Otsuka A, Tanizaki H, Minegaki Y, Nagao K, Waldmann H et al. Enhanced murine contact hypersensitivity by depletion of endogenous regulatory T cells in the sensitization phase. J Dermatol Sci. 2011;61(2):144-7. https://doi.org/10.1016/j.jdermsci.2010.11.001
18. Engebretsen KA, Thyssen JP. Skin barrier function and allergens. Curr Probl Dermatol. 2016;49:90-102. https://doi.org/10.1159/000441548

19. Hogan D, Ledet JJ. Impact of regulation on contact dermatitis. Dermatol Clin. 2009;27(3):385-94. https://doi.org/10.1016/j.det.2009.05.006

20. Gerberick GF, House RV, Fletcher ER, Ryan CA. Examination of the local lymph node assay for use in contact sensitization risk assessment. Fundam Appl Toxicol. 1992;19(3):438-45. https://doi.org/10.1016/0272-0590(92)90183-I

21. Montelius J, Wahlkvist H, Boman A, Fernström P, Gråbergs L, Wahlberg JE. Experience with the murine local lymph node assay: inability to discriminate between allergens and irritants. Acta Derm Venereol. 1994;74(1):22-7.

22. Basketter DA. REVIEW Skin sensitization: risk assessment. Int J Cosmet Sci. 1998;20(3):141-50. https://doi.org/10.1046/j.1467-2494.1998.171746.x

23. Levin CY, Maibach HI. Irritant contact dermatitis: is there an immunologic component? Int Immunopharmacol. 2002;2(2-3):183-9. https://doi.org/10.1016/S1567-5769(01)00171-0

24. Slodownik D, Lee A, Nixon R. Irritant contact dermatitis: a review. Australas J Dermatol. 2008;49(1):1-9. https://doi.org/10.1111/j.1440-0960.2007.00409.x

25. Tüzün Y, Antonov M, Dolar N, Wolf R. Keratinocyte cytokine and chemokine receptors. Dermatol Clin. 2007;25(4):467-76. https://doi.org/10.1016/j.det.2007.06.003

26. Watanabe H, Unger M, Tuvel B, Wang B, Sauder DN. Contact hypersensitivity: the mechanism of immune responses and T cell balance. J Interferon Cytokine Res. 2002;22(4):407-12. https://doi.org/10.1089/10799900252952181

27. Effendy I, Löffler H, Maibach HI. Epidermal cytokines in murine cutaneous irritant responses. J Appl Toxicol. 2000;20(4):335-41. https://doi.org/10.1002/1099-1263(200007/08)20:4<335::AID-JAT698>3.0.CO;2-L

28. Eberhard Y, Ortiz S, Ruiz Lascano A, Kuznitzky R, Serra HM. Up-regulation of the chemokine CCL21 in the skin of subjects exposed to irritants. BMC Immunol. 2004;5:7. https://doi.org/10.1186/1471-2172-5-7

29. Lee HY, Stieger M, Yawalkar N, Kakeda M. Cytokines and chemokines in irritant contact dermatitis. Mediators Inflamm. 2013;2013:ID916497. https://doi.org/10.1155/2013/916497

30. Gerberick GF, Cruse LW, Ryan CA, Hulette BC, Chaney JG, Skinner RA et al. Use of a B cell marker (B220) to discriminate between allergens and irritants in the local lymph node assay. Toxicol Sci. 2002;68(2):420-8. https://doi.org/10.1093/toxsci/68.2.420

31. Jung KM, Jang WH, Lee YK, Yum YN, Sohn S, Kim BH et al. B cell increases and ex vivo IL-2 production as secondary endpoints for the detection of sensitizers in non-radioisotopic local lymph node assay using flow cytometry. Toxicol Lett. 2012;209(3):255-63. https://doi.org/10.1016/j.toxlet.2011.12.012

32. Lee JK, Hee Park S, Byun JA, Kim HS, Oh HY. Evaluation of lymphocyte subpopulations in draining lymph node cells following allergen and irritant. Environ Toxicol Pharmacol. 2004;17(2):95-102. https://doi.org/10.1016/j.etap.2004.03.006

33. Neves BM, Cruz MT, Francisco V, Gonçalo M, Figueiredo A, Duarte CB et al. Differential modulation of CXCR4 and CD40 protein levels by skin sensitizers and irritants in the FSDC cell line. Toxicol Lett. 2008;177(1):74-82. https://doi.org/10.1016/j.toxlet.2007.12.006

34. Goutet M, Pépin E, Langonné I, Huguet N, Ban M. Identification of contact and respiratory sensitizers using flow cytometry. Toxicol Appl Pharmacol. 2005;205(3):259-70. https://doi.org/10.1016/j.taap.2004.10.011

35. Silva O, Perez MJ, Pineau N, Rougier A, Dossou KG. Local lymph node assay: study of the in vitro proliferation and control of the specificity of the response by FACScan analysis. Toxicol In Vitro. 1993;7(4):299-303. https://doi.org/10.1016/0887-2333(93)90018-Z

36. Battais F, Huppert C, Langonné I, Muller S, Sponne I. In vitro detection of chemical allergens: an optimized assay using mouse bone marrow-derived dendritic cells. Contact Dermat. 2017;77(5):311-22. https://doi.org/10.1111/cod.12829

37. Asherson GL, Barnes RM. Classification of immunological unresponsiveness and tolerance. Proc R Soc Med. 1973;66(5):468-71.

38. Maurer T. Guinea pigs in hypersensitivity testing. Methods. 2007;41(1):48-53. https://doi.org/10.1016/j.ymeth.2006.06.017

39. Chung H, Quan H, Jung D, Ravi G, Cho A, Kang M et al. Intra- and inter-laboratory reproducibility and predictivity of the HaCaSens assay: a skin sensitization test using human keratinocytes, HaCaT. Toxicol Vitr. 2018;46:304-12. https://doi.org/10.1016/j.tiv.2017.10.018

40. Vliet E, Kühnl J, Goebel C, Martinozzi-Teissier S, Alépée N, Ashikaga T et al. State-of-the-art and new options to assess T cell activation by skin sensitizers: Cosmetics Europe Workshop. ALTEX. 2017;35(2):179-92. https://doi.org/10.14573/altex.1709011

41. Wareing B, Urbisch D, Kolle SN, Honarvar N, Sauer UG, Mehling A et al. Prediction of skin sensitization potency sub-categories using peptide reactivity data. Toxicol In Vitro. 2017;45(Pt 1):134-45. https://doi.org/10.1016/j.tiv.2017.08.015

42. Wang J, Suárez-Fariñas M, Estrada Y, Parker ML, Greenlees L, Stephens G et al. Identification of unique proteomic signatures in allergic and non-allergic skin disease. Clin Exp Allergy. 2017;47(11):1456-67. https://doi.org/10.1111/cea.12979

43. Buehler EV. Delayed contact hypersensitivity in the guinea pig. Arch Dermatol. 1965;91:171-7. https://doi.org/10.1001/archderm.1965.01600080079017

44. Magnusson B, Kligman AM. The identification of contact allergens by animal assay. The guinea pig maximization test. J Invest Dermatol. 1969;52(3):268-76. https://doi.org/10.1038/jid.1969.42

45. Och FM, Vandebriel RJ, Prinsen MK, De Jong WH, Slob W, Loveren H. Comparison of dose-responses of contact allergens using the guinea pig maximization test and the local lymph node assay. Toxicology. 2001;167(3):207-15. https://doi.org/10.1016/S0300-483X(01)00425-5

46. Kimber I, Hilton J, Weisenberger C. The murine local lymph node assay for identification of contact allergens: a preliminary evaluation of in situ measurement of lymphocyte proliferation. Contact Dermatitis. 1989;21(4):215-20. https://doi.org/10.1111/j.1600-0536.1989.tb03198.x

47. Kreiling R, Hollnagel HM, Hareng L, Eigler D, Lee MS, Griem P et al. Comparison of the skin sensitizing potential of unsaturated compounds as assessed by the murine local lymph node assay (LLNA) and the guinea pig maximization test (GPMT). Food Chem Toxicol. 2008;46(6):1896-904. https://doi.org/10.1016/j.fct.2008.01.019

48. Omori T, Idehara K, Kojima H, Sozu T, Arima K, Goto H et al. Interlaboratory validation of the modified murine local lymph node assay based on adenosine triphosphate measurement. J Pharmacol Toxicol Methods. 2008;58(1):11-26. https://doi.org/10.1016/j.vascn.2008.05.001

49. Strickland J, Zang Q, Kleinstreuer N, Paris M, Lehmann DM, Choksi N et al. Integrated decision strategies for skin sensitization hazard. J Appl Toxicol. 2016;36(9):1150-62. https://doi.org/10.1002/jat.3281

50. Organization for Economic Cooperation and Development - OECD. OECD Guidelines for testing of chemicals, section 4: test no. 406: skin sensitisation. 1992[acesso 20 nov 2017]. Disponível em: https://www.oecd-ilibrary.org/environment/test-no-406-skin-sensitisation_9789264070660-en

51. Organization for Economic Cooperation and Development - OECD. Skin sensitisation: local lymph node assay. Paris: Organization for Economic Cooperation and Development; 2002. (OECD Guideline for the testing of chemicals, 429).

52. Kimber I, Weisenberger C. A murine local lymph node assay for the identification of contact allergens. Assay development and results of an initial validation study. Arch Toxicol. 1989;63(4):274-82. https://doi.org/10.1007/BF00278640

53. Hans-Werner V, Jürgen AH. The local lymph node assay being too sensitive? Arch Toxicol. 2005;79(12):721-8. https://doi.org/10.1007/s00204-005-0005-9

54. Yamashita K, Shinoda S, Hagiwara S, Miyazaki H, Itagaki H. Unsaturated fatty acids show clear elicitation responses in a modified local lymph node assay with an elicitation phase, and test positive in the direct peptide reactivity assay. J Toxicol Sci. 2015;40(6):843-53. https://doi.org/10.2131/jts.40.843

55. Arancioglu S, Ulker OC, Karakaya A. Utilization of the ex vivo LLNA: BrdU-ELISA to distinguish the sensitizers from irritants in respect of 3 end points-lymphocyte proliferation, ear swelling, and cytokine profiles. Int J Toxicol. 2015;34(1):24-30. https://doi.org/10.1177/1091581814560613

56. Roberts DW, Api AM, Aptula AO. Chemical applicability domain of the Local Lymph Node Assay (LLNA) for skin sensitisation potency. Part 2. The biological variability of the murine Local Lymph Node Assay (LLNA) for skin sensitisation. Regul Toxicol Pharmacol. 2016;80:255-9. https://doi.org/10.1016/j.yrtph.2016.07.013

57. Organization for Economic Cooperation and Development - OECD. Skin sensitization: local lymph node assay. Paris: Organization for Economic Cooperation and Development; 2010. (OECD Guideline for testing of chemicals, 4442a).

58. Organization for Economic Cooperation and Development - OECD. Sensitization: local lymph node assay: BrdU-ELISA. Paris: Organization for Economic Cooperation and Development; 2010. (OECD Guideline for testing of chemicals, 442b)

59. EU: final ban on animal experiments for cosmetic ingredients implemented. ALTEX. 2013;30(2):268-9.

60. Organization for Economic Cooperation and Development - OECD. Guidance document on the reporting of defined approaches and individual information sources to be used within integrated approaches to testing and assessment (IATA) for skin sensitisation. Paris: Organization for Economic Cooperation and Development; 2016.

61. Organization for Economic Cooperation and Development - OECD. Chemico skin sensitization: Direct Peptide Reactivity Assay (DPRA). Paris: Organization for Economic Cooperation and Development; 2010. (OECD Guideline for testing of chemicals, 442c)

62. Organization for Economic Cooperation and Development - OECD. In vitro skin sensitisation : ARE-Nrf2 Luciferase test method.). Paris: Organization for Economic Cooperation and Development; 2010. (OECD Guideline for testing of chemicals, 442d)

63. Pamies D, Hartung T, Barroso J, Casey W, Delgado I, Kim TS et al. Standardisation of defined approaches for skin sensitisation testing to support regulatory use and international adoption: position of the International Cooperation on Alternative Test Methods. Arch Toxicol. 2018;92(2):611-7. https://doi.org/10.1007/s00204-017-2097-4

64. Pamies D, Hartung T. 21st Century cell culture for 21st Century Toxicology. Chem Res Toxicol. 2017;30(1):43-52. https://doi.org/10.1021/acs.chemrestox.6b00269

65. Hartung T. Utility of the adverse outcome pathway concept in drug development. Expert Opin Drug Metab Toxicol. 2017;13(1):1-3. https://doi.org/10.1080/17425255.2017.1246535

66. Hartung T, Hoffmann S, Stephens M. Mechanistic validation. ALTEX. 2013;30(2):119-30. https://doi.org.10.14573/altex.2013.2.119

67. Póvoa TF, Oliveira ERA, Basílio-de-Oliveira CA, Nuovo GJ, Chagas VLA, Salomão NG et al. Peripheral organs of dengue fatal cases present strong pro-inflammatory response with participation of IFN-Gamma-, TNF-Alphaand RANTES-producing cells. PLoS One. 2016;11(12):e0168973.

68. O'Leary JG, Goodarzi M, Drayton DL, Andrian UH. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. Nat Immunol. 2006;7(5):507-16. https://doi.org/10.1038/ni1332

69. Larsen JM, Geisler C, Nielsen MW, Boding L, Von Essen M, Hansen AK et al. Cellular dynamics in the draining lymph nodes during sensitization and elicitation phases of contact hypersensitivity. Contact Dermat. 2007;57(5):300-8. https://doi.org/10.1111/j.1600-0536.2007.01230.x

70. Ku HO, Jeong SH, Kang HG, Pyo HM, Cho JH, Son SW, et al. Gene expression profiles and pathways in skin inflammation induced by three different sensitizers and an irritant. Toxicol Lett. 2009;190(2):231-7. https://doi.org/10.1016/j.toxlet.2009.07.022

71. Cortés JR, Sánchez-Díaz R, Bovolenta ER, Barreiro O, Lasarte S, Matesanz-Marín A et al. Maintenance of immune tolerance by Foxp3 regulatory T cells requires CD69 expression. J Autoimmun. 2014;55:51-62. https://doi.org/10.1016/j.jaut.2014.05.007

72. Homey B, Schilling C, Blümel J, Schuppe HC, Ruzicka T, Ahr HJ et al. An integrated model for the differentiation of chemical-induced allergic and irritant skin reactions. Toxicol Appl Pharmacol. 1998;153(1):83-94. https://doi.org/10.1006/taap.1998.8535

73. Strauss V, Kolle SN, Honarvar N, Dammann M, Groeters S, Faulhammer F et al. Immunophenotyping does not improve predictivity of the local lymph node assay in mice. J Appl Toxicol. 2015;35(4):434-45. https://doi.org/10.1002/jat.3042

74. Tuschl H, Landsteiner HT, Kovac R. Application of the popliteal lymph node assay in immunotoxicity testing: complementation of the direct popliteal lymph node assay with flow cytometric analyses. Toxicology. 2002;172(1):35-48. https://doi.org/10.1016/S0300-483X(01)00583-2

75. Jung KM, Bae IH, Kim BH, Kim WK, Chung JH, Park YH et al. Comparison of flow cytometry and immunohistochemistry in non-radioisotopic murine lymph node assay using bromodeoxyuridine. Toxicol Lett. 2010;192(2):229-37. https://doi.org/10.1016/j.toxlet.2009.10.024

76. Basketter D, Darlenski R, Fluhr JW. Skin irritation and sensitization: mechanisms and new approaches for risk assessment. Skin Pharmacol Physiol. 2008;21(4):191-202. https://doi.org/10.1159/000135635

77. Lee JK, Park JH, Kim HS, Chung ST, Eom JH, Nam KT et al. Evaluation of cell proliferation in ear and lymph node using BrdU immunohistochemistry for mouse ear swelling test. Environ Toxicol Pharmacol. 2003;14(1-2):61-8. https://doi.org/10.1016/S1382-6689(03)00025-5

78. Dubois B, Chapat L, Goubier A, Papiernik M, Nicolas JF, Kaiserlian D. Innate CD4+CD25+ regulatory T cells are required for oral tolerance and inhibition of CD8+ T cells mediating skin inflammation. Blood. 2003;102(9):3295-301. https://doi.org/10.1182/blood-2003-03-0727

Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.