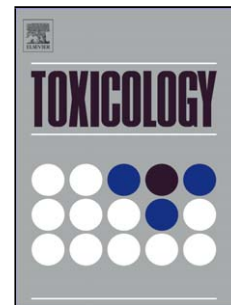


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Simultaneous analysis of the local and systemic immune responses in mice to study the occupational asthma mechanisms induced by chromium and platinum.

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Abstract

As a result of industrial development, increased exposure to platinum and chromium compounds and the subsequent development of occupational asthma (OA) have been reported. Although specific IgE antibodies, an indicator of allergic asthma, against chromium and platinum have been detected in workers with OA, the immunopathological mechanisms involved in this disease are not fully understood. To better understand these complex mechanisms, the local and systemic immune responses were simultaneously analyzed in mice sensitized and challenged 3, 4, or 5 times with sodium hexachloroplatinate (Pt salt) and with potassium dichromate (Cr salt) via the respiratory route. Dinitrochlorobenzene (DNCB) and anhydride trimellitic (TMA) were included in this study as reference compounds that induce Th1 and Th2 responses respectively.

All the compounds studied may provoke pulmonary sensitization. In the Pt salt treated mice with a significant increase in local Th2 cytokine production, the increase in IgE and mucus production and in eosinophil number had a positive correlation with the number of challenges ($r = 0.942, 0.976, \text{ and } 0.978$ respectively), whereas in the Cr salt treated mice with no local increase in Th2 cytokines, the increase in IgE production and eosinophil numbers had an inverse correlation with the number of challenges ($r = -0.895$ and -0.999 respectively). The Th2-dominated response induced by Pt salt was very close to that induced by TMA and may thrive after the fifth challenge, probably due to the constancy of the significant decrease in IFN- γ level in the spleens.

The results of the present work may increase our understanding of the immunopathological mechanisms of OA induced by platinum and chromium, and emphasize the advantage of simultaneously analyzing local and systemic immune response when studying respiratory allergy.

Key words: Occupational asthma, platinum and chromium exposure, Th2-dominated

1. Introduction

Metals and their derivatives, considered as low molecular weight (LMW) compounds, are in widespread use in the workplaces of industrialized countries. As a result of industrial development, increased exposure to platinum and chromium compounds has been reported in the platinum refining industry (Bolm-Audorff et al., 1992; Cristaudo et al. 2005), cement plants (WHO, 1998) and welding workshops (Hannu et al., 2005) respectively. A number of studies have shown that both platinum and chromium compounds may cause allergic occupational asthma (OA) with IgE antibody-mediated immune response (Cromwell et al., 1979; Boggs 1985; Novey et al. 1983). However, the immunopathological mechanisms by which these compounds mediate IgE response are not fully understood. Dearman et al. (1998) demonstrated that topical exposure of mice to platinum salts elicited a Th2-type quality of immune responses. On the other hand, chromium salts are known to induce a mixed Th1- and Th2-type cytokine production in topically exposed mice as well as in peripheral blood mononuclear cells from individual patients with contact allergy to chromium (Minang et al. 2006). While cutaneous dermatitis resulting from exposure to chromium compounds is well documented (Forte et al. 2008; Dayan and Paine, 2001), there are only few reports relative to Th2-mediated respiratory sensitization (Novey et al. 1983; Park et al. 1994).

The skin and pulmonary epithelium represent a primary barrier preventing the entry of exposed compounds into the body. However, depending on several factors such as the intrinsic nature of chemicals, the microenvironment, and the cytokine/chemokine levels at the sites of deposited chemicals, many LMW substances may mediate epithelium inflammatory reaction through mechanisms which are not totally understood (Akdis et al. 2009). In general, LMW substances are reactive and act as haptens. These substances can only be recognized as antigens after they combine with proteins of the host to form immunologic conjugates. An immune sensitization is then caused by the uptake of these conjugates by antigen-presenting cells within the skin or the airways. This is followed by priming of allergen-specific CD4⁺ T cells in the draining lymph nodes, i.e., in the lung-associated lymph nodes when the respiratory tract is the exposure route. After antigen presentation, CD4⁺ T cells coordinate immune responses by producing a range of cytokines and are divided into different effector T helper (Th) cell subsets based on the functional profiles and cytokines produced (Akdis and Akdis 2009; Létourneau et al. 2009). Concerning Th1/Th2 cell subsets, Th1 cells typically producing large amounts of IFN- γ and IL-2 skew the immune response towards Th1 cell-mediated immune response, whereas Th2 cells producing large amount of IL-4, IL-5, and IL-

13 skew the immune response towards humoral Th2 immune response (Mosmann et al. 1986). The balance between Th1 and Th2 cytokines may be a key event in determining the type of immune response induced by sensitizing agents, and is not fixed. Indeed the development of Th2 cells followed by the synthesis of IL-4 and IL-13 (Th2 dominant response) may be down regulated in certain experimental conditions by the increase in Th1 type cytokine IFN- γ (Kang et al. 2009).

There is evidence that OAs provoked by high-molecular weight protein are mainly of a selective Th2 type response, whereas OAs induced by LMW compounds may be a Th1 or Th2 dominant response or a Th1/Th2 mixed response (Bauer, 1995; Maestrelli et al. 2009). This however remains highly debatable because of the presence or absence of antigen specific IgE antibody detected in some LMW exposed workers with asthma (Karol et al. 1994). There have been experimental studies aimed at identifying and enhancing the understanding of LMW compound-induced allergic immune response via the respiratory tract. Pauluhn et al. (2008) suggested that the inhalation route should be given preference when trying to identify chemical-induced respiratory sensitization and allergy, and that repeated challenge exposure may aggravate preceding events, allowing a conclusive and robust assessment. Arts et al. (2008), using the respiratory local lymph nodes as a tool to study chemical respiratory sensitizers, concluded that contact allergens are as potent sensitizers as respiratory allergens via respiratory tract. In our previous work, which examined the local cytokine profile in association with different modes of sensitization and challenge, we showed that, depending on the experimental conditions, toluene diisocyanate can induce a Th2 dominant immune response in mice (Ban et al. 2006a). At the present time, however, there are few, if any, studies simultaneously investigating both local and systemic Th1/Th2 cytokine profiles in animals sensitized and challenged by LMW compounds via the respiratory tract. Recently, Yamada et al. (2009) reported that a Chinese herbal medicine preparation inhibited the development of peanut allergy in the intestine of mice due to the higher number of IFN- γ producing T cells in the spleen, i.e., systemic response. Thus, the data from local and systemic cytokines may yield additional information concerning Th1/Th2 balanced responses following exposure to LMW respiratory sensitizing agents like metallic salts.

The purpose of the present work was to better understand the complexity of the Th2 immune mechanisms involved in the occurrence of OA in workers exposed to chromium and platinum. To do this, the simultaneous analysis of local and systemic cytokine production was investigated in mice sensitized and challenged with potassium dichromate (Cr salt) or sodium hexachloroplatinate (Pt salt). Other respiratory endpoints such as lung histology,

inflammatory cell influx in the bronchoalveolar lavage (BAL) fluid, intracellular IL-4 and its CD124 receptor, and serum total IgE levels were also investigated to highlight the cytokine results. On the other hand, two others LWM compounds, trimellitic anhydride (TMA) and dinitrochlorobenzene (DNCB), known as allergic respiratory and skin sensitizers respectively, were included in this study as models for Th1 or Th2 dominant immune responses (Friedman et al. 1983; Grammer et al. 2002)

2. Materials and methods

2.1. Chemicals

The sodium hexachloroplatinate (Pt salt), TMA, and DNCB were purchased from Aldrich (France), whereas the potassium dichromate (Cr salt) was purchased from Merck. For dermal application, TMA (10%) and DNCB (1%) were dissolved in 4:1 acetone/ olive oil. For intratracheal instillation, TMA (1 %) and DNCB (0.1 %) were dissolved in 1:9 acetone/ olive oil. Concerning the metallic compounds, i.e. Cr and Pt salts, both were dissolved in saline solution (0.3%; W/V) for the intradermal injections. For the intranasal instillations, the concentrations were twofold lower (0.15 %). These concentrations were chosen on the basis of the results of preliminary experiments showing that these concentrations could increase the serum total IgE without causing either lesions at the application sites or the death of the animal.

2.2. Mice

Female, 8 to 10 week-old BALB/c mice were obtained from Charles River (Italy). The animals were housed in an environmentally controlled animal facility maintained at 22 °C with a 12h-12h light/dark cycle. They were provided with food and water ad libitum for the duration of the experiment. For each chemical tested, four groups of 12 mice were used: one control group and three treated groups, designated 3-challenged group, 4-challenged group and 5-challenged group.

2.3. Experimental design in the main study

The experimental design was similar to that described in the previous study (Ban et al. 2006a).

Briefly, to sensitize the mice on day 0, the three treated groups received two intradermal injections of metallic salt solution (50 μ L of fresh solution per mouse) in each shaved flank, and on day 3 an intranasal instillation of 50 μ L of the same type of fresh metallic solution but with a twofold lower concentration. To challenge the mice, the 3-challenged group received three intranasal instillations with the same concentration as the former instillation on days 17, 24 and 34. The 4-challenged group received the same challenged condition as the 3-challenged group, except for an additional challenge on day 10, and the 5-challenged group received the same challenge conditions as the 3-challenged group, except for two additional challenges on day 10 and 31. The control group received the same treatment conditions as the 5-challenged group, but with only a salt solution. With respect to the reference compounds, TMA and DNCB were administered in accordance with the aforementioned protocol except that the intradermal injections and the intranasal instillation were replaced by cutaneous application and intratracheal instillation respectively. 1 % DNCB and 10 % TMA in acetone/olive oil vehicle (4:1; V/V) were used for cutaneous application and 0.1 % DNCB and 1 % TMA in acetone/olive oil (1:9; V/V) were used for intratracheal instillation. The reasons for the change of administration mode are twofold: 1) as the chemicals were dissolved in an acetone/ olive oil vehicle, cutaneous application is more reliable than intradermal injection, 2) to reach the lower respiratory tract, which is drained by the lung-associated lymph nodes, chemicals in oil must be administered by an intratracheal instillation (Ebino et al. 1999).

The skin and the respiratory tract are important routes of exposure and sites of sensitization to allergens. In this work the experimental design using lung-associated lymph nodes after dermal and respiratory administration of metallic salts may be a realistic approach to study the occupational asthma mechanisms. Due to the timing of cell preparation, however, the limitation of experimental design in the main study is such that only a pool of lung-associated lymph nodes per group of mice was carried out

2.4. Experimental design in the additional study

In this part of the study, the experimental design was identical to that of the main study, except for the number of mice and groups. These three-challenge experiments were designed

to back up and confirm certain results obtained in the main study by using a flow cytometric method or by increasing the lymph node pool number in each group of mice for the statistical analyze of the results.

2.5. Sample collection

The samples were collected under intraperitoneal pentobarbital anesthesia (60mg/Kg body weight).

On day 35, six mice per group were sacrificed and the spleens and lung-associated lymph nodes removed after blood sampling by cardiac puncture. For the cell culture in the main study, unlike the spleen, the lung-associated lymph nodes were pooled to obtain sufficient cell numbers (one pool /group). However, in the additional study, to confirm the significant difference in cytokine levels between the treated and the control groups, five or six pools of lung-associated lymph nodes per group were produced, the pools being obtained from two mice instead of six.

On day 36, the rest of the mice were sacrificed. After bronchoalveolar lavage (BAL) of the entire lungs, the left lobe of the lungs was removed for histology.

2.6. Cell cultures

- Lymph node cell culture

As mentioned above, the lung-associated lymph nodes were pooled. Single cell suspensions were prepared by gently mashing the lymph nodes through a sterile 70µm cell strainer (Falcon) in cold RPMI-1640 medium (Gibco) supplemented with 10 % fetal calf serum (Biowest), 2 mM L-glutamine, 100 U/ml penicillin and 100µg/ml streptomycin (Gibco). After washing by centrifugation, the cell number was adjusted to 2×10^6 cells/ml, and 5×10^5 cells/well (250µl) were seeded on plastic 96-well plates with ConA (3µg/ml; Sigma) and cultured for 72 h at 37 °C in a controlled atmosphere: 14 % oxygen, 7 % carbon dioxide and balanced nitrogen. For each group, the culture supernatants of 10 identical cultures were collected, pooled and stored at -80 °C until used to assess the cytokine levels.

- Spleens

The spleen was individually mashed without lysing the erythrocytes. The cell preparation and culture technique was identical to that described above for the lung-associated lymph nodes,

but with a few modifications: after washing, the nucleated cell number was adjusted to 5×10^6 cells/ml, and 7.5×10^6 cells/well (1.5ml) were seeded on plastic 24-well plates (Falcon).

2.7. Serum total IgE and cytokine analysis

The serum total IgE and the Th1/Th2 cytokines were analyzed using ELISA kits (IgE and IL-5 set from Pharmingen and IL-4, IL-13 and IFN- γ from R&D Systems, France) according to the manufacturers' protocols. Briefly, 96-well microimmunoplates (NUNC) were coated with capture antibody and incubated overnight before washing with 0.1-M phosphate-buffered saline (PBS) containing 0.05 % of Tween-20 (Wash buffer). After 2 h incubation at room temperature with the samples (sera or culture supernatant), the wells were washed with wash buffer. The biotinylated antibody and streptavidin conjugated to peroxidase were then added. After repeated washing in wash buffer, the plates were incubated with peroxidase substrate and the reaction was stopped by adding 2N H₂SO₄. Optical density was measured at 450nm with a micro plate reader (Lab System, Multiskan, MS).

2.8. BAL fluid analysis

After cardiac puncturing, the BAL fluid was collected by cannulating the trachea and lavaging the lung four times with 0.3 ml (each time) of sterile PBS. For the differential cell counts, 100 μ l ($\sim 6 \times 10^4$ cells) of the suspended cells were spun (cytospin) on microscope slides, air-dried and Diff-Quik stained. From each slide, at least 200 cells were differentiated by means of conventional morphologic criteria.

2.9. Lung histology

Two slides per mouse were prepared by sectioning the left lobe of the lungs (6 μ m) along the main stem bronchus. One cryostat section was stained by means of the periodic acid-Schiff method to identify airway mucous (goblet) cells and the other was stained using the "Diff-Quik" method to highlight the influx of inflammatory cells around the airways. The relative degree of severity of inflammatory cell influx and proliferative change was semi-quantitatively assessed by blindly scoring from 0 to 4. For cell influx around the airways and vessels: score 0 (no inflammatory cell); score 1 (a few inflammatory cells); score 2 (scattered aggregates of inflammatory cells); score 3 (thin layer of inflammatory cells surrounding the

airways and vessels); score 4 (thick layer of inflammatory cells surrounding the airways and vessels). For goblet cell proliferation: score 0 (positive goblet cells $\leq 5\%$); score 1 (positive goblet cells $\leq 20\%$); score 2 (positive goblet cells $\leq 35\%$); score 3 (positive goblet cells $\leq 50\%$); score 4 (positive goblet cells $> 50\%$). The results were expressed as the means of the numerical scores.

2.10. Cytometry investigation

The following monoclonal antibodies (mAbs) to mouse cell surface antigens or intracellular cytokines and matched isotype controls were obtained from BD-Pharmingen (Pont-de-Claix, France): anti-CD3e-Cy-Chrome (clone 145-2C11), anti-CD4-Cy-Chrome (clone H129.19), anti-CD8a-fluorescein-isothiocyanate (FITC) (clone 53-6.7), anti-CD19-R-Phycoerythrin (PE) (clone 1D3), anti-CD124-PE (clone Mil 4R-M1), anti-IgE-FITC (clone R35-72), anti-CD16/CD32 (FcBlock) (clone 2.4G2), and anti-IL-4-PE (clone BVD4-1D11).

- Cell surface staining and IL-4 investigation

The cell surface staining of CD3, CD4, CD8, CD19, CD124 and IgE and the determination of intracellular IL-4 were performed on fresh lymph node cells with specific mAbs, as previously described (Goutet et al. 2004). The pooled lymph node cells of the treated and control mice were obtained from three repeated experiments.

- Flow cytometry analysis

Simultaneous three-colour analysis of cells was performed using a Becton Dickinson FACStar^{PLUS} flow cytometer equipped with an argon 488-laser (*INNOVA EnterpriseTM II*) controlled by the CELLQuest software tool. Fluorescence intensities and compensations were calibrated using singularly stained samples. For the cell surface staining analysis, the forward and side scatter properties of the lymphocytes were used to gate the viable lymphocytes and exclude erythrocytes, dead cells and debris from the analysis. For the IL-4 analysis, the lymphocytes were dual gated on physical parameters (forward and side scatter) and on positive fluorescence for CD4 or CD8. At least 20,000 gated lymphocytes were analyzed per sample. The analyses were performed using the CELLQuest software. The percentages of positive cells were calculated after setting the threshold in accordance with the isotype controls. CD124 molecule expression was assessed by means of the relative fluorescence intensity (RFI) calculated by the following formula: $RFI = (\text{mean fluorescence intensity})$

(MFI) of lymphocytes from allergen treated mice / MFI of lymphocytes from control mice) x 100.

3. Data analysis

One-way analysis of variance (ANOVA) followed by multiple group comparison (Duncan's post hoc test) was used to analyze the data. When the variance was heterogeneous, the data were log-transformed. Significance was set at ($p < 0.05$).

3. Results

3.1. Main study

- IL-4

The IL-4 levels in the lymph node cell cultures are shown in Figure 1A.

The IL-4 levels of the Cr salt-treated mice, whatever the number of challenges, decreased compared with those of the control mice. On the contrary, the IL-4 levels of the Pt salt-treated mice receiving 3 challenges increased more than threefold those of the controls. Increased IL-4 levels were also observed in the culture of the TMA- treated mice. In the spleen cell cultures (Fig.1B), both Cr salt and Pt salt caused a statistically significant decrease in IL-4 level.

DNCB caused a statistically significant IL-4 decrease in the spleen cell cultures of the after five challenges.

- IL-5

The IL-5 levels in the lymph node cell cultures are shown in Figure 2A.

In contrast to the Cr salt treatment, which caused a decrease in IL-5 level in the cell cultures, the Pt salt treatment caused an increase in IL-5 levels which was approximately 2 to 2.5 times higher than the control level. In the spleen cell cultures (Fig. 2B), significantly lower IL-5 levels were observed in the cultures of the DNCB or Cr salt-sensitized mice undergoing 5 challenges and in those of the Pt salt-sensitized mice with 4 challenges. In the TMA treated mice, the IL-5 level was similar to that of the controls.

- IL-13

The IL-13 levels in the lymph node cell cultures are shown in Figure 3A.

In contrast to the Cr salt treatment, which tended to reduce IL-13 production in the lymph node cell cultures, the TMA, DNCB and Pt salt treatments resulted in higher IL-13 levels. In the Pt salt-treated mice with 3 challenges, the level was five times that of the controls. In the spleen cell cultures (Fig.3B), all the compounds studied caused a decrease in the IL-13 levels. Statistically significant decreases were observed in the cell cultures of the DNCB and Cr salt treated mice with five challenges, and in those of the Pt salt- treated mice with four or five challenges.

- IFN- γ

The IFN- γ levels in the lymph node cell cultures are shown in Figure 4A.

The IFN- γ levels in the lymph node cell cultures of the treated mice fluctuated around the control values, except for that of the Pt salt- treated mice with 5 challenges, which was up to 1.7 times lower. In the spleen cell cultures (Fig.4B), significant decreases in IFN- γ level were observed in the cultures of the TMA or Pt salt- treated mice with four or five challenges. As for the Cr salt treated mice, a statistically significant increase was only observed after 4 challenges.

- Total serum IgE

All the compounds studied caused a statistically significant increase in IgE level compared with their control values (Fig. 5). In the Pt salt and TMA mice, there was a positive correlation between the higher IgE levels and the number of challenges ($r = 0.974$ and 0.972 respectively). A 6.5 and 8 fold higher increase was observed in the TMA and Pt salt treated mice respectively. In the DNCB and Cr salt treated mice, however, there was a negative correlation between higher IgE level and challenge number ($r = - 0.969$ and $- 0.895$ respectively).

- BAL fluid neutrophil number

The neutrophil percentages in the BAL fluids are shown in Fig. 6A.

All the compounds caused a neutrophil influx into the BAL fluids. In the Cr salt and Pt salt-treated mice, 3 or 4 challenges caused a significant neutrophil increase; up to 45 % in the Pt salt mice with 4 challenges. However, 5 challenges alleviated the neutrophil influx. A significant neutrophil increase was also observed in the DNCB-treated mice with 3 challenges.

- BAL fluid eosinophil number

The eosinophil percentages in the BAL fluids are shown in Fig. 6B.

Pt and Cr salt caused an eosinophil influx into the BAL fluids. In the Pt salt mice there was a positive correlation between higher eosinophils and challenge number ($r = 0.978$), whereas in the Cr salt mice a negative correlation was observed ($r = -0.999$).

- Lung histological section: inflammatory scores

The inflammatory cell influx scores (0 to 4) are shown in Fig. 7A.

All the compounds caused an inflammatory cell influx into the lung and around the airways and vessels. Compared with the scores of control mice, a statistically significant inflammatory cell influx score was found only in the Cr and Pt salt treated mice.

- Lung histological section: hyperplasia scores

The airway goblet cell hyperplasia scores (0 to 4) are shown in Fig. 7B.

In the Cr salt, DNCB and TMA- treated mice, the scores were similar to those of the controls, whereas in the Pt salt mice the hyperplasia scores (up to 4) were significantly higher than those of the controls and had a positive correlation with the number of challenges ($r = 0.976$).

The representative pictures of lung inflammation and goblet cell hyperplasia are shown in Fig. 8.

3.2. Additional study

3.2.1. Increased Th2 cytokines in lymph node cell cultures

To confirm whether the higher IL-4, IL-5 and IL-13 levels were statistically significant, five to six pools of lymph nodes per group of mice were investigated in the additional study (Fig. 9). In the lymph node cell cultures of the Pt salt- treated mice, the IL-4, IL-5 and IL-13 cytokine levels were significantly higher than those of the control, whereas in the cultures of the TMA- treated mice a significant increase in IL-4 level alone was observed.

3.2.2. Cytometric analysis of the lung-associated lymph node cells

- Membrane IgE expression

The percentages of IgE+CD19+B cells with membrane IgE are shown in Fig. 10A, derived from one representative experiment.

In the control mice, the percentage of B cells in the CD19+ IgE+ B cells was barely 1 %, whereas in the Pt and Cr salt-treated mice the B- cell population increased by approximately 35 % and 14 % B cells respectively.

- Intracellular IL-4 production

The percentages of IL-4 positive CD4+ T cells are shown in Fig. 10B, derived from one representative experiment.

In the Pt salt-treated mice, approximately 1 % of CD4⁺ T cells expressing IL4⁺ were observed, whereas in the control or Cr salt-treated mice this cell population did not exceed 0.1 %.

- Expression of IL-4 receptor CD124

Three histograms from three independent experiments revealing the CD124 membrane expressed by CD3⁺T cells are highlighted in Fig.10C.

In the Pt salt-treated mice, the lymph node cells exhibited up-regulation of CD124 expression with a RFI value reaching 1.54, whereas in both the Cr salt-treated mice and the control mice no up-regulated CD124 expression was observed (RFI value: 1.07).

4- Discussion

Specific IgEs against chromium and platinum in asthmatic workers have been reported in the literature (Novey et al. 1983; Cromwell et al. 1979), the assumption being that these metals and their derivatives may cause allergic asthma mediated by a Th2 immune response. Previously, we and other investigators (Fukuyama et al. 2008; Ban et al. 2006a and 2006b; Vanoirbeek et al. 2006) have assessed an *ex vivo* cytokine production in ConA- or anti-CD3-stimulated local lymph node cell cultures to explain an *in vivo* event in a murin asthma model. These studies demonstrated a relation between the Th2-cytokine production and the pulmonary allergic inflammation including a polymorphonuclear cell influx in alveolar spaces and pulmonary tissue, which persists several days after an allergen challenge. In the present work we mimicked the previously established experimental design to better understand the underlying asthma mechanisms induced by chromium or platinum salts. To this end, groups of mice were sensitized and challenged according to the experimental design described in materials and methods. The analyses, cytokine assays and inflammation assessment, were achieved one day and two days after the last challenge, respectively. As the influx of inflammatory cells depends partly on the cytokines present in the lungs, we thought that the 24 h delay observed between both analyses would allow to better characterize the relation between the synthesis of cytokines and the inflammation in the lungs.

In this study, both metallic salts and both reference substances caused pulmonary sensitization, as evidenced by the significant increase in the total IgE levels and the augmentation of inflammatory cell influx in and around the airways. However, the IgE production in the TMA and Pt salt treated mice was greater than that in the DNCB and Cr salt mice, the highest levels being reached in mice undergoing 5 challenges. In the DNCB and Cr

1 salt-treated mice, five challenges caused relatively weaker IgE production than that observed
 2 in those undergoing 3 or 4 challenges. Furthermore, in the TMA and Pt salt mice, the much
 3 higher IgE levels coincided with the significant increase in Th2 cytokine production,
 4 particularly IL-4, by the lung-associated lymph node cells, whereas lower Th2 cytokine levels
 5 were observed in the Cr salt mice. IL-4 is the key cytokine in IgE production, as previously
 6 mentioned in the literature (Kaplan et al 1996; Oettgen, 2000), and in this work the higher IgE
 7 production, which depended on the number of challenges, can probably be explained by local
 8 IL-4 production. On account of the lower Th2 cytokine levels in the spleen cell cultures
 9 observed in the TMA and Pt salt mice and in the DNCB and Cr salt mice, the results of this
 10 study support the idea that the local production of Th2 cytokines, particularly IL-4, is the
 11 overriding mechanism in the higher IgE production observed in the serum of the TMA and Pt
 12 salt mice.
 13

14 With respect to IL-5 and IL-13, the higher level of these cytokines found in asthmatic
 15 subjects is responsible for promoting the development, recruitment, activation and survival of
 16 inflammatory eosinophil cells and for airway goblet cell proliferation respectively (Foster et
 17 al. 2002; Xiang et al. 2008). In this study, the goblet cell hyperplasia scores in the Pt salt mice
 18 were significantly higher than in the control mice and coincided with the higher IL-13 level
 19 produced by the lymph node cells, whereas in the Cr salt mice, where the IL-13 produced by
 20 the lymph node cells tended to decrease, these score were similar to the controls. There may
 21 be a link between increased IL-13 production and goblet proliferation in these mice.
 22 Concerning IL-5, this study did not allow a clear explanation for the link between IL-5 level
 23 and airway-space eosinophil influx. Indeed, in the Cr salt mice undergoing three or four
 24 challenges a significantly higher eosinophil number was observed in the absence of lymph
 25 node cell produced IL-5. However, in this work, the manner in which eosinophil increased,
 26 whatever the IL-5 level, may differentiate the immune response induced by Cr salt from that
 27 induced by Pt salt. In the Pt salt mice, where the higher IL-5 level was observed, the
 28 eosinophil number became significant after 5 challenges, whereas in the Cr salt mice 5
 29 challenges reduced the eosinophil number down to the base line.
 30

31 An increase in IFN- γ production, which is a signature cytokine for a Th1 type immune
 32 response, has been observed in Th-2 driven allergic asthma (Akkoc et al.2008; William et al.
 33 2006). In this study based on the IFN- γ produced by lymph node cells, the quality of the
 34 immune responses induced by Pt or by Cr salt could not be differentiated, although in the Pt
 35 salt-treated mice undergoing 5 challenges the IFN- γ level was almost half the control value,
 36 and in the Cr salt mice the level was similar to the control level. However, the results of the
 37

IFN- γ produced by the spleen cells may shed some light on this divergence. Indeed, the comparison between TMA and DNCB treatment revealed a significantly lower IFN- γ level in the spleen cell cultures of the TMA-treated mice, whereas this level was similar to the controls in the case of the DNCB-treated mice. On the other hand, the comparison of Pt and Cr salt treatment highlighted that the Pt salt treated mice undergoing the additional challenge retained a significantly lower IFN- γ level. This contrasts with the Cr salt mice, where 5 challenges raised the IFN- γ level. The results of the amount of IFN- γ produced by spleen cells led to our speculating that there may be communication taking place between the lymph nodes and the spleen and that the Th2 predominated immune response caused by TMA or by Pt salt is partly the result of the lower production of IFN- γ by spleen cells, leaving room for the Th2 cell polarization to occur. Further studies will however be needed to confirm these results. Recently, Mitchell and colleagues (2009) suggested that signals from the lung can activate signals in the spleen to suppress the immune function of mice exposed to inhaled multiwalled carbon nanotubes.

The Th2-dominated response observed in the main study, an indicator of allergic asthma, induced by Pt salt was confirmed by the results obtained in the additional study. Unlike Cr salt, Pt salt sensitization provoked a significant increase in the IL-4, IL-5, and IL-13 levels of the lymph node cell culture as well as a rise in the number of lymph node cells with intracellular IL-4 and up-regulation of the IL-4 receptors CD124. The intracellular IL-4 and its receptors could be a further clue to discriminating the immune response induced by Pt salt from that by Cr salt. The conclusive results of the IgE, eosinophil, goblet cell hyperplasia, and systemic IFN- γ were obtained after 5 challenges. According to these results, the maximal number of challenges with Cr or Pt salts might not develop an immune tolerance but rather orient the response towards a selective type of immune response, through an interaction between a local and systemic cytokine network.

The results of the present work highlight the different immune mechanisms induced by Pt and by Cr salt and the interest in simultaneously investigating lung associated-lymph node and spleen cells when studying the respiratory sensitization potential of chemicals administered by the respiratory route.

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Legends

Fig.1. The IL-4 levels in the culture supernatant were measured by ELISA. (A) the data concerning the IL-4 level in the lymph node cell cultures are represented as the mean $\pm$ S.D.

of two measurements. (B) the data concerning the IL-4 level in the spleen cell cultures were represented as the mean $\pm$ S.E.M. of 6 mice.

* significantly different from controls $p < 0.05$.

Fig.2.. The IL-5 levels in the culture supernatant were measured by ELISA. (A) the data concerning the IL-5 level in the lymph node cell cultures were represented as the mean $\pm$ S.D. of two measurements. (B) the data concerning the IL-5 level in the spleen cell cultures were represented as the mean $\pm$ S.E.M. of 6 mice.

* significantly different from controls $p < 0.05$.

Fig.3. The IL-13 levels in the culture supernatant were measured by ELISA. (A) the data concerning the IL-13 level in the lymph node cell cultures were represented as the mean $\pm$ S.D. of two measurements. (B) the data concerning the IL-13 level in the spleen cell cultures were represented as the mean $\pm$ S.E.M. of 6 mice.

* significantly different from controls $p < 0.05$.

Fig.4. The IFN- γ levels in the culture supernatant were measured by ELISA. (A) the data concerning the IFN- γ level in the lymph node cell cultures were represented as the mean $\pm$ S.D. of two measurements. (B) the data concerning the IFN- γ in the spleen cell cultures were represented as the mean $\pm$ S.E.M. of 6 mice.

* significantly different from controls $p < 0.05$.

Fig.5. The serum total IgE levels of the control and the sensitized mice undergoing, 4 and 5 challenges were measured by ELISA. Data were represented as the mean $\pm$ S.E.M. of 5 or 6 mice.

* significantly different from controls $p < 0.05$.

Fig.6. The lungs of the control and the sensitized mice receiving 3, 4, and 5 challenges were lavaged for 48 hours after the last challenge. Different cell types were counted after cytopspin preparation of the bronchoalveolar lavage fluid and Diff-Quik staining.

Data were represented as the mean $\pm$ S.E.M. percentage of 5 or 6 mice; (A) neutrophil percentage and (B) eosinophil percentage.

* significantly different from controls $p < 0.05$.

Fig.7. The left lung of the control and sensitized mice receiving 3, 4, and 5 challenges were removed 48 hours after the last challenge. The histological scores (0 to 4) of pulmonary inflammation were obtained from 6 μ m cryostat sections stained with Diff-Quik. Data represented as the mean $\pm$ S.E.M. of 5 or 6 mice; (A) inflammatory cell influx scores and (B) goblet cell hyperplasia scores.

* significantly different from controls $p < 0.05$.

Fig.8. Histopathology of lungs from controls (A, B) and Cr or Pt salt sensitized mouse (C- F). Tissue sections (6 μ m) were stained with "Diff- Quik" solution (A, C, and E) or with periodic acid-Schiff (B, D, and F). There were infiltrations of inflammatory cells around airways caused by Cr salt or Pt salt (C and E, arrowhead) and goblet cell hyperplasia in airways caused by Pt salt (E, arrow). Pictures were taken at 20x magnification.

Fig.9. The pooled lymph node cells (5×10^5 cells/well) were seeded on plastic 96-well plates with ConA ($3\mu\text{g/mL}$) for 72 hours. The IL-4, IL-5 and IL-13 levels in the culture supernatant were measured by ELISA. Data were represented as the mean $\pm$ S.E.M. of 5 or 6 cultures of pooled lymph node cells. Left; Th2 cytokine levels of the control and sensitized mice receiving 3 challenges with Pt salt. Right; idem with TMA.

* significantly different from controls $p < 0.05$.

Fig.10. Cytometry analysis of the pooled lymph node cells from the control, the Cr salt-treated and the Pt salt-treated mice. (A) percentage of the CD19+IgE+ positive cells in one representative experiment. (B) Percentage of the CD4+ IL-4+ positive cells in one representative experiment is shown. (C) membrane expression of the cells with IL-4 receptor CD124. The control mice are represented by the shaded areas, the Cr salt-treated mice by the dotted lines, and the Pt salt-treated mice by the full lines. The three graphs of the membrane expression of CD124 are representative of three repeated experiments.

Fig. 1

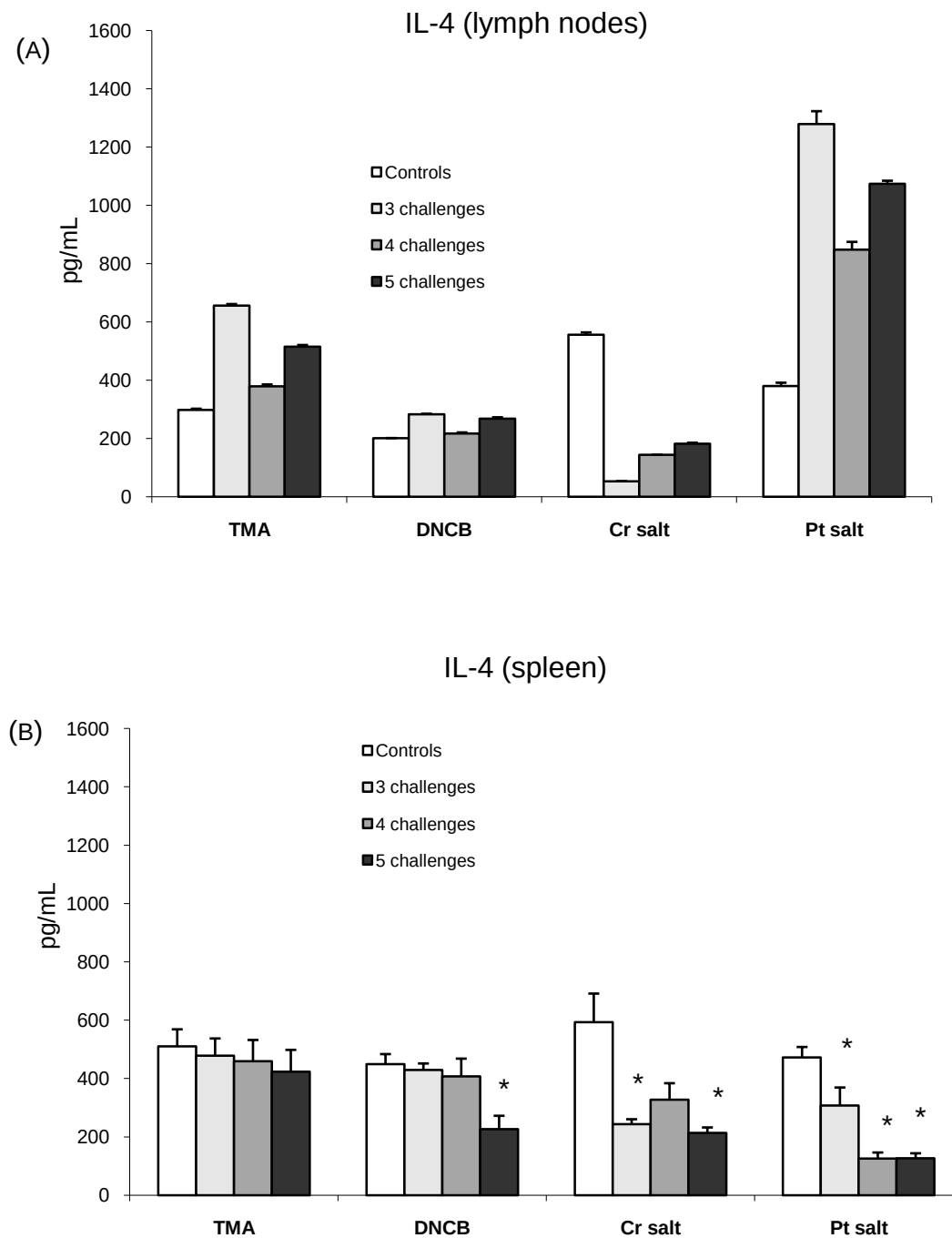


Fig.2

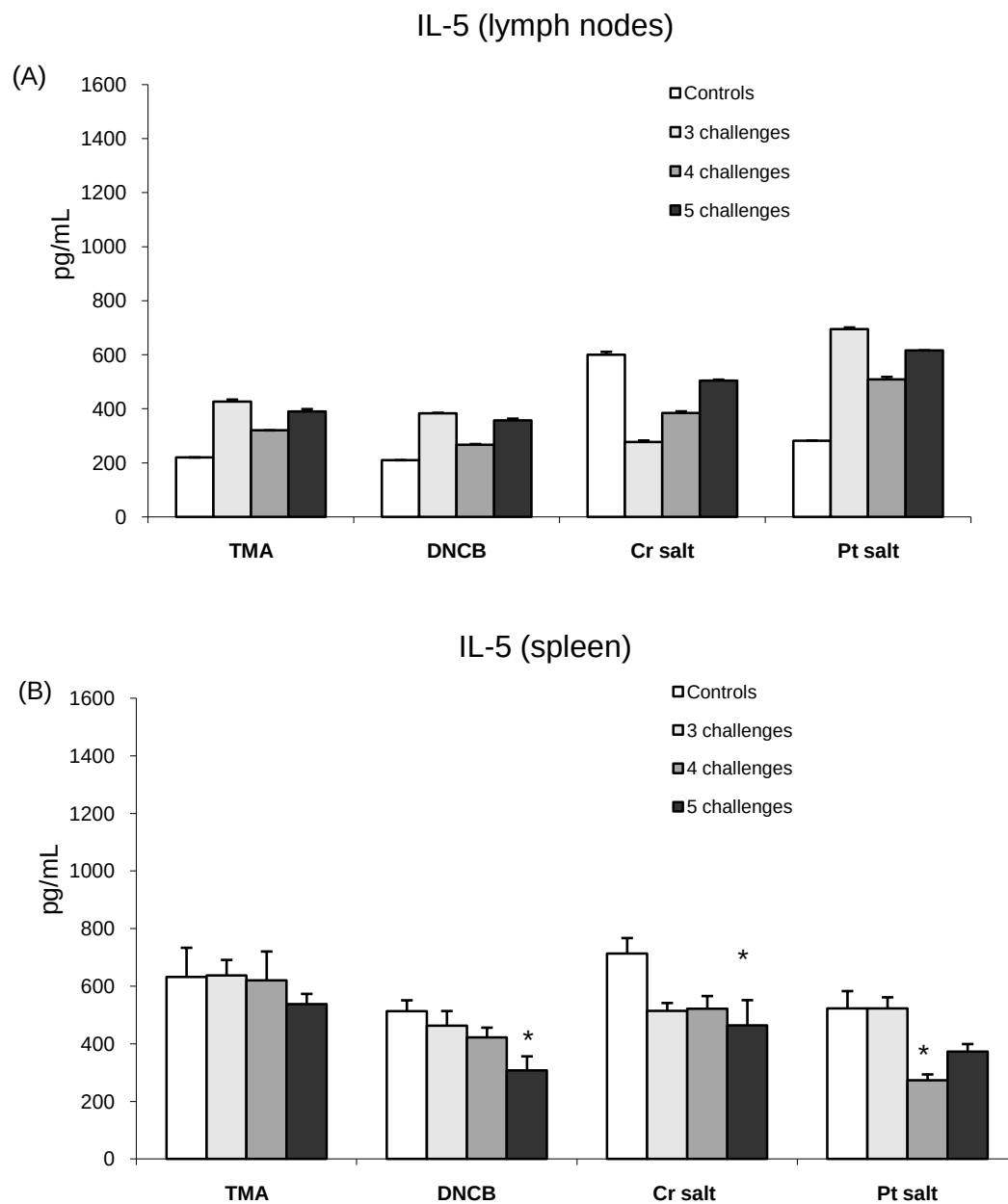


Fig.3

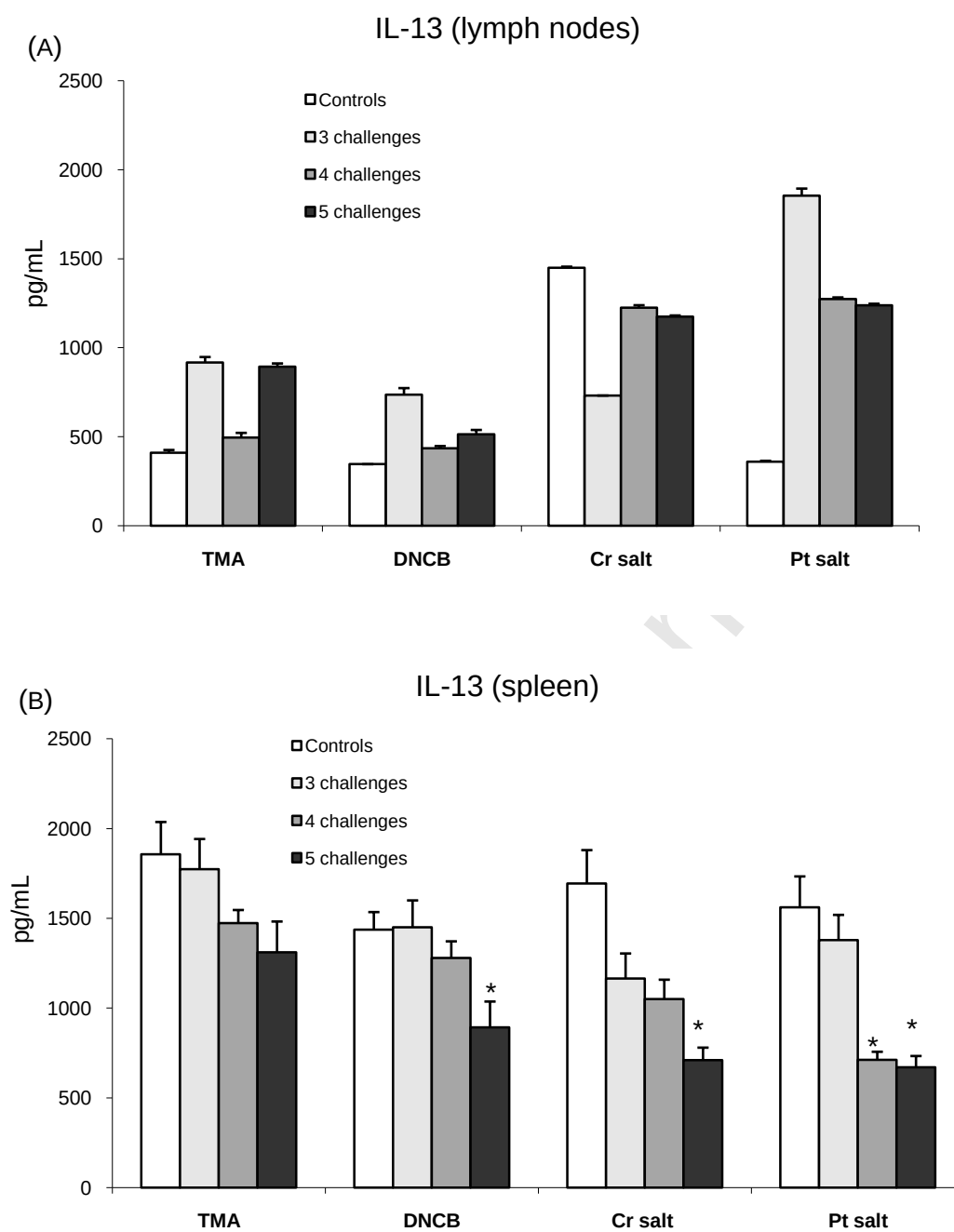


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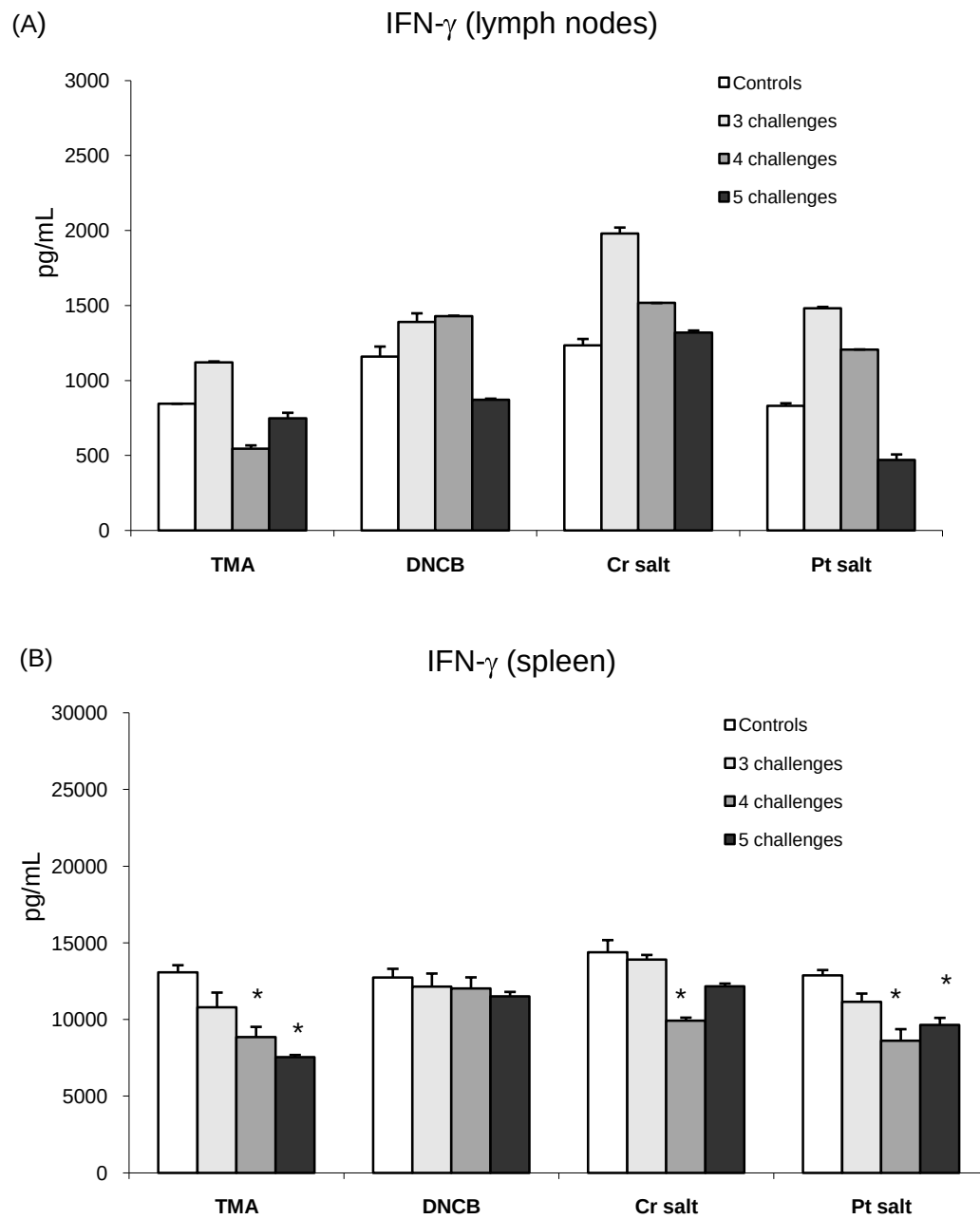


Fig. 5

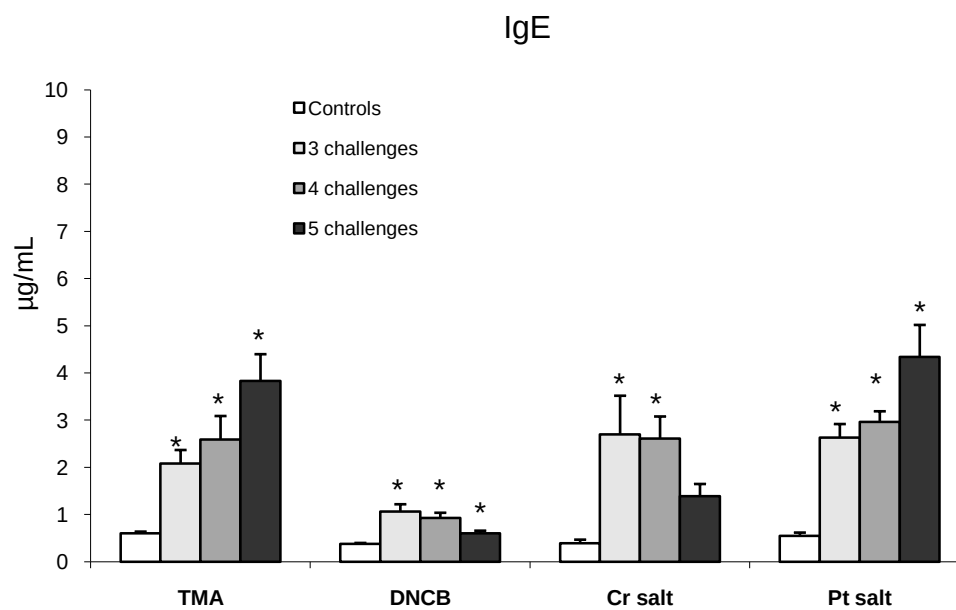


Fig. 6

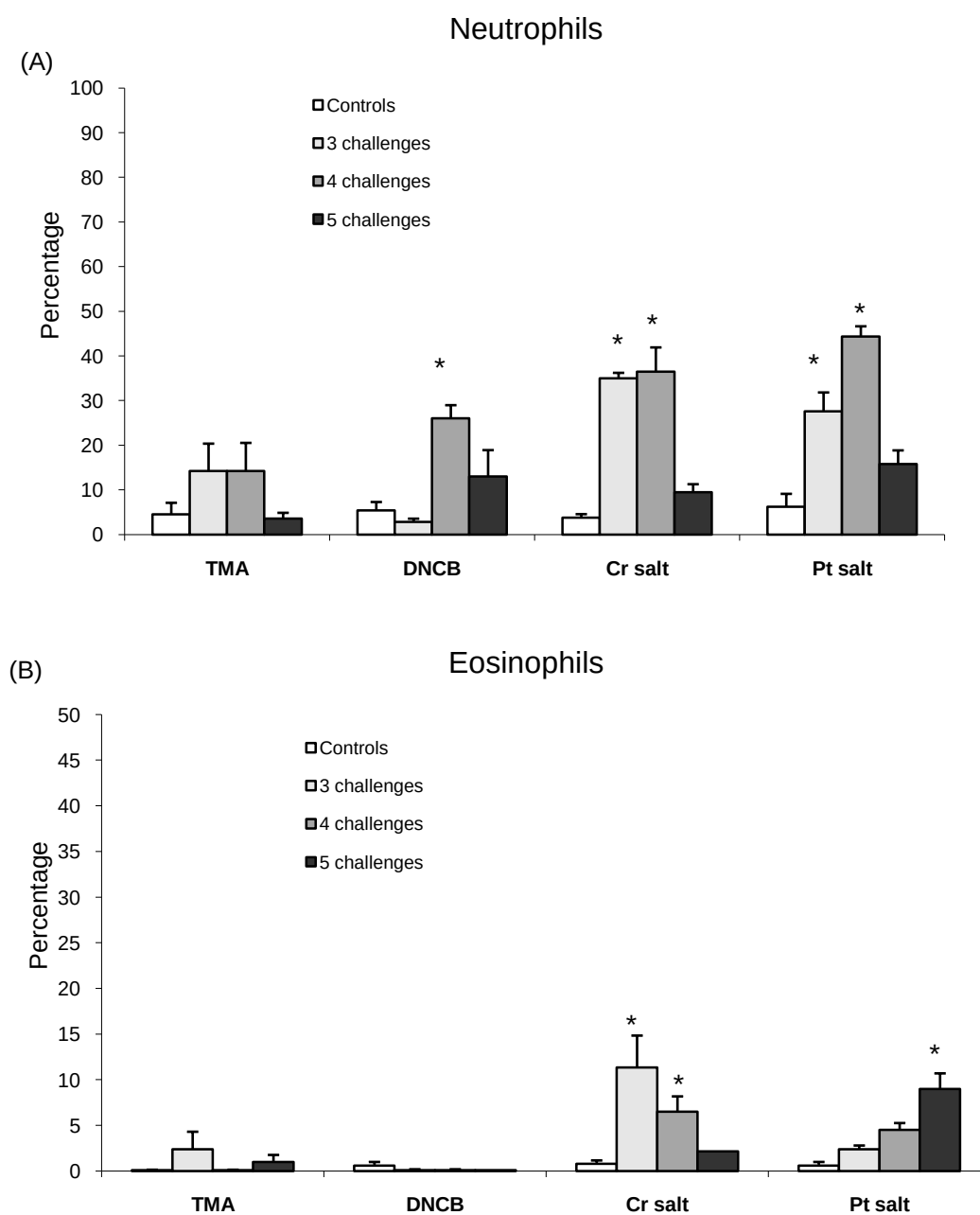
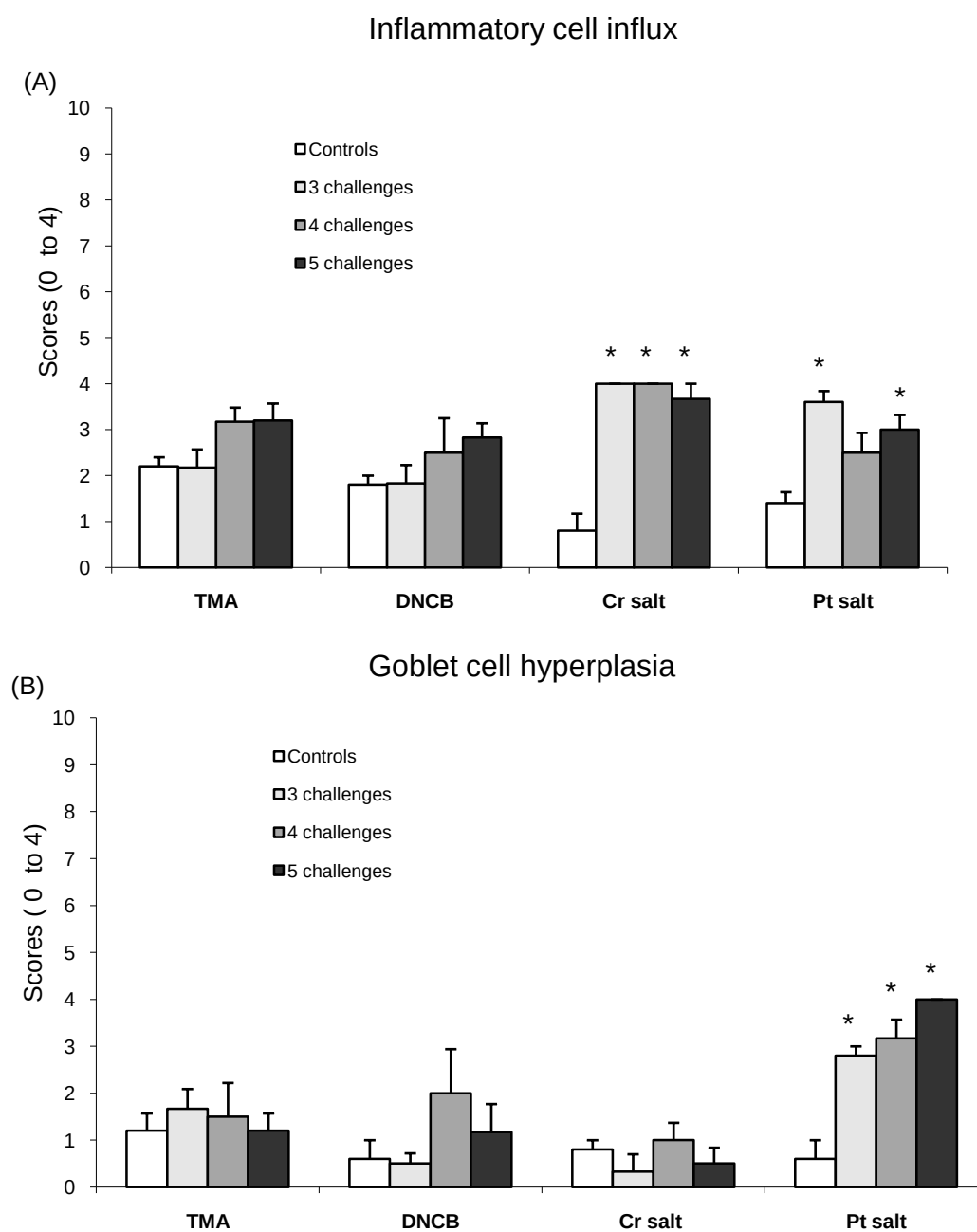


Fig. 7



Figure

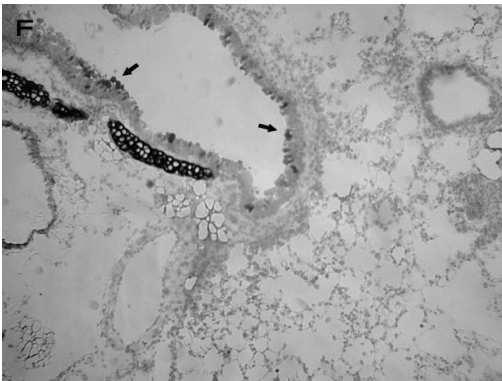
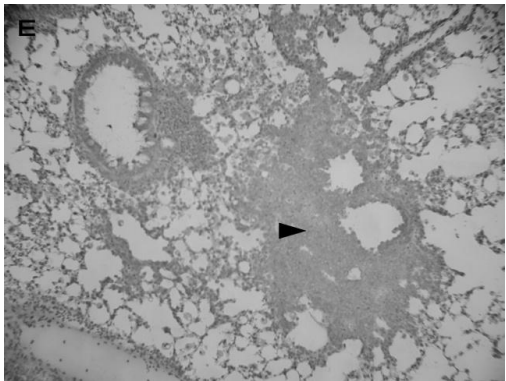
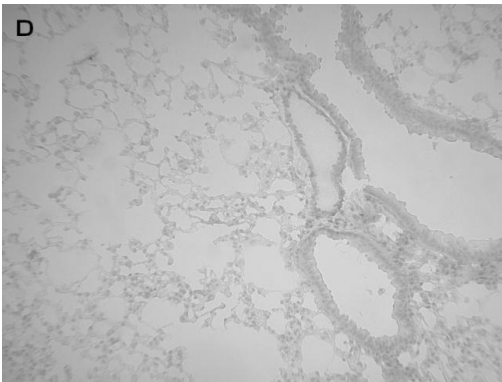
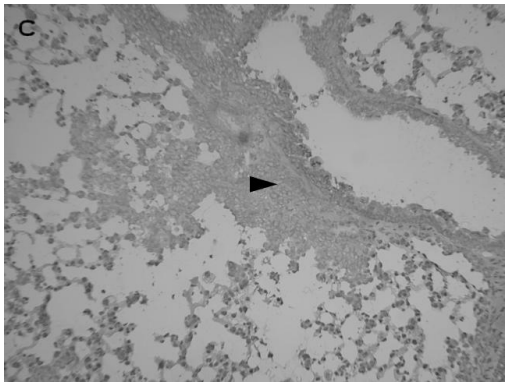
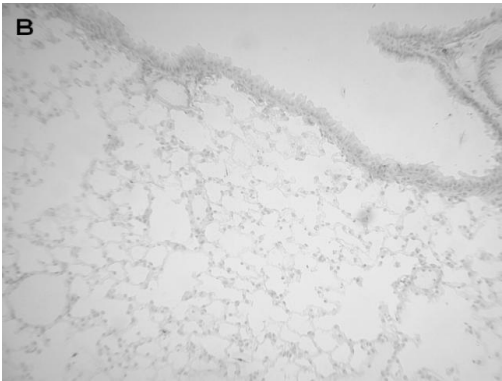
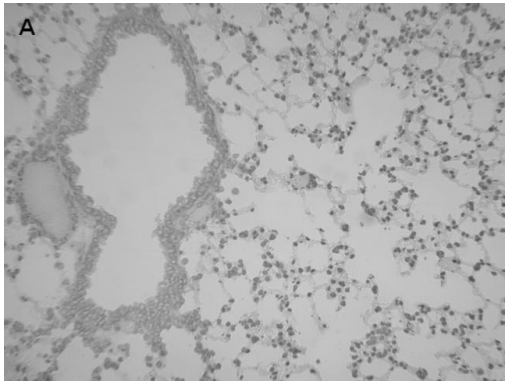


Fig. 9

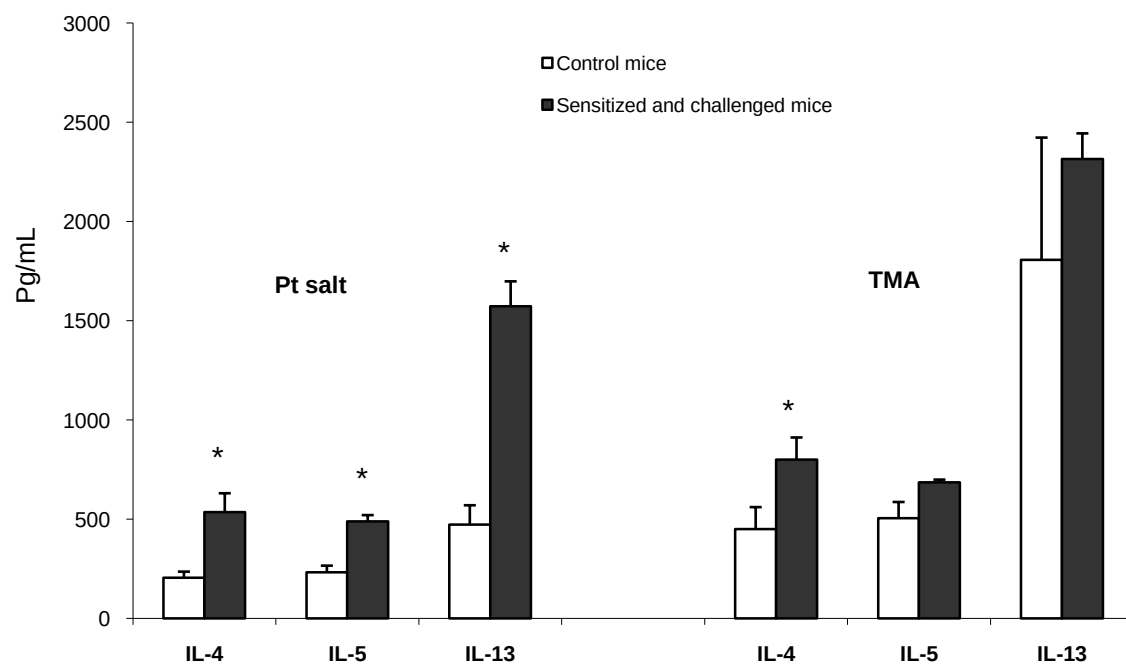
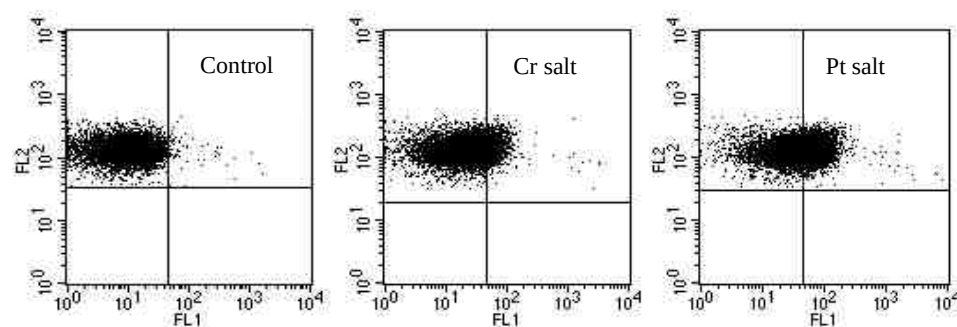
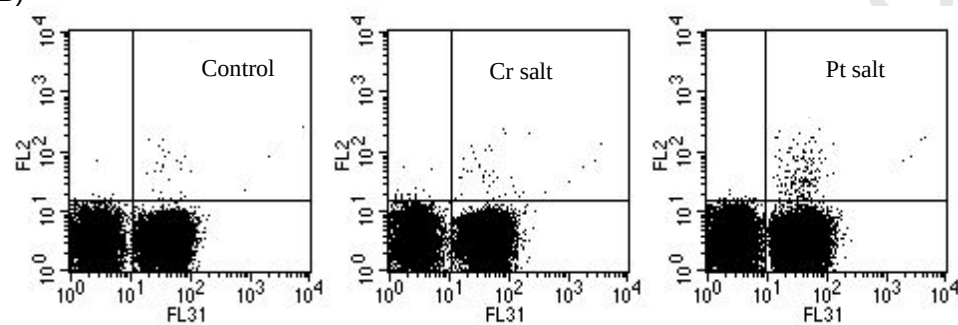


Fig.10

(A)



(B)



(C)

