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Multiple challenges in a mouse model of chemical-induced asthma lead to tolerance: Ventilatory and inflammatory responses are blunted, immunologic humoral responses are not[☆]

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ABSTRACT

To improve our mouse model of chemical-induced asthma we compared a single with a multiple intranasal challenge protocol.

BALB/c mice received toluene diisocyanate (TDI) or vehicle on each ear (days 1 and 8) with the first challenge by intranasal instillation given on day 15. In a “long” protocol, the mice received 1 to 6 intranasal instillations, with 1-week interval. In a “short” protocol, the mice received 6 intranasal challenges over a period of 10 days. The “early” ventilatory response and methacholine reactivity were measured. Bronchoalveolar lavage (BAL), total serum immunoglobulins and draining lymph nodes were analyzed.

After 1, 2 or 3 TDI challenges, a significant increase in airway reactivity, total cell count and neutrophils (15–20%) was found in TDI-treated mice. This response diminished with increasing numbers of challenges in both models. The percentage CD4⁺ and CD8⁺ cells decreased and the percentage CD19⁺ cells increased in the lymph nodes, but these returned to control values with multiple challenges. IL-4 secretion increased in cervical lymph node cells *in vitro*. Total serum IgE levels were persistently increased in TDI-treated mice.

Although humoral signs of allergy remain increased after multiple challenges, diminishing ventilatory and inflammatory responses are indicative of the induction of tolerance.

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1. Introduction

Asthma is a chronic respiratory disease characterized by reversible airway obstruction, non-specific airway hyper-responsiveness and airway inflammation (Lemanske and Busse, 2003). The prevalence of asthma is increasing, reaching epidemic proportions. Approximately 9–15% of adult-onset asthma is considered to be caused by occupational exposures, but the population incidence may be underestimated by 50% (Boulet et al., 2007). Isocyanates, reactive chemicals used in the production of polyurethane

foams, coatings, and a wide array of consumer products, are a major cause of occupational asthma worldwide (Bello et al., 2007).

Occupational asthma, is caused by a particular occupational environment and has become the most important cause of work-related lung diseases in the industrial world (Chan-Yeung and Malo, 1995). This is caused in part by the introduction of new biological and chemical products in agriculture and industry. Compounds capable of causing occupational asthma are divided into high-molecular mass (HMM) and low-molecular mass (LMM) agents. HMM agents induce asthma by immunoglobulin (Ig)E-mediated mechanisms, while LMM components have to bind to endogenous proteins and are believed to act through IgE-mediated or non-IgE-mediated mechanisms (Chan-Yeung and Malo, 1995; Mapp et al., 2005).

We have developed a mouse model for chemical-induced asthma (Tarkowski et al., 2007; Vanoirbeek et al., 2003, 2004, 2006b, 2008). Following dermal sensitization, a single intranasal instillation leads to an antigen-specific early ventilatory response, increased airway responsiveness to methacholine 24 h later, along with increased inflammatory markers (neutrophils) in bronchoalveolar lavage (BAL). These responses depend on the presence of lymphocytes since no response is found in severe combined immunodeficiency disease (SCID) mice (Tarkowski et al., 2007).

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In order to improve our mouse model of chemical-induced asthma and yield a more pronounced asthmatic response, we evaluated two different multiple exposure regimes. In the first study, we hypothesized that repeated challenges (1–6 intranasal challenges), with 1-week interval, would yield an inflammation characterized by eosinophils, lymphocytes and neutrophils, instead of solely neutrophils. However, contrary to our hypothesis, our results showed a diminishing airway response and lung inflammation with multiple intranasal challenges. This led us to a second hypothesis, i.e. that an intense (shorter) exposure protocol would maintain the airway responses and inflammation and would not result in the development of 'tolerance'. In this second part we also investigated whether the time point of methacholine provocation and autopsy (1, 2, 3 or 4 days) is critical for measuring respiratory responses, lung inflammation and immunological parameters.

2. Materials and methods

2.1. Reagents

Toluene-2,4-diisocyanate (98%; Fluka, CAS584-84-9), acetyl- β -methylcholine (methacholine), acetone and diethyl ether were obtained from Sigma-Aldrich (Bornem, Belgium). Pentobarbital (Nembutal®) was obtained from Sanofi Santé Animale (CEVA, Brussels, Belgium). The vehicle (AOO) used to dissolve TDI consisted of a mixture of 2 v of acetone and 3 v of olive oil (Selection de Almazara, Carbonell, Madrid, Spain). Concentration of TDI is given as percent (v/v) in AOO.

2.2. Animals

Male BALB/c mice (approximately 20 g, 6 weeks old) were obtained from Harlan (Horst, The Netherlands). The mice were housed in a conventional animal house with 12-h dark/light cycles. They were housed in plastic cages with filter tops and received lightly acidified water and pelleted food (Trouw Nutrition, Gent, Belgium) *ad libitum*. All experimental procedures were approved by the local Ethical Committee for Animal Experiments.

2.3. Groups of animals and treatment protocol

Fig. 1A and B shows the two different treatment protocols. In both protocols, on days 1 and 8 the animals were dermally treated with 0.3% TDI or vehicle applied on the dorsum of both ears (20 μ l). In the first (long) protocol, from day 15 on, mice received, under light diethyl ether anaesthesia, 1–6 intranasal instillations (10 μ l/nos-tril) of 0.1% TDI (challenge) or vehicle, with 1-week interval between instillations. Mice were sacrificed 24 h after the last challenge. In the second (short) protocol, on days 15, 16, 17, 22, 23 and 24 they received under light diethyl ether anaesthesia, an intranasal instillation of 10 μ l vehicle or 10 μ l 0.1% TDI in each nostril. Each group (vehicle or TDI treated) consisted of 10 animals of which 5 undergo a methacholine provocation using the whole body plethysmography followed by an autopsy, while the other 5 did not undergo methacholine provocation before sacrifice.

2.4. Ventilatory function

2.4.1. Early ventilatory response and airway hyper-reactivity

In the long protocol, before and after each intranasal challenge the ventilatory function was recorded, while in the short protocol, only before and after

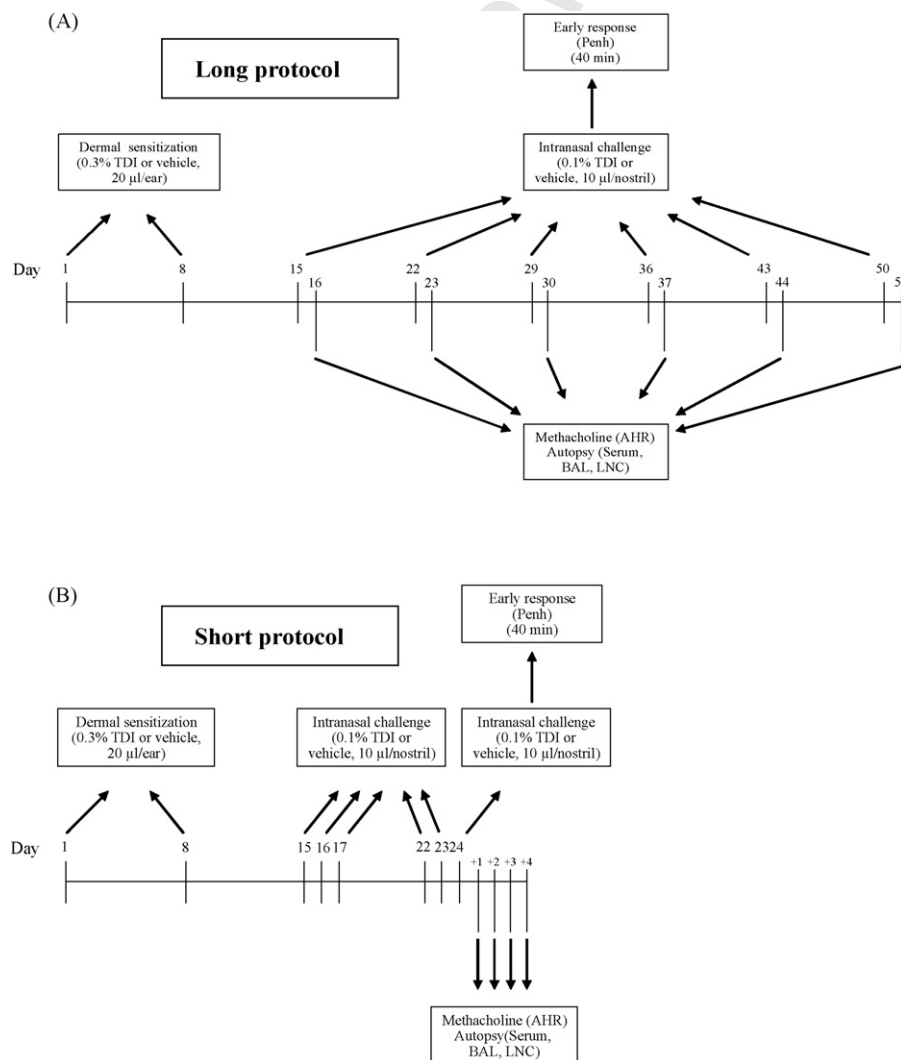


Fig. 1. Treatment and endpoints of the long (A) and the short (B) challenge protocol. AHR: airway hyper-reactivity; BAL: broncho-alveolar lavage; LNC: lymph node cells; Penh: enhanced pause; R: airway resistance; TDI: toluene diisocyanate; vehicle: acetone–olive oil (2:3); WBP: whole body plethysmography.

the last challenge this measurement was performed. The measurement of ventilatory function was performed as follows: first the ventilatory function of each mouse was recorded, in resting condition and unrestrained, for 5 min in a whole body plethysmograph (EMKA Technologies, Paris, France). Immediately after the intranasal instillation, mice were placed again in the whole body plethysmograph (zero time point) and their ventilatory parameters were measured for 40 min. Every 30 s, the mean enhanced pause (Penh), which is a composite index indicative of airway obstruction, was calculated. The area under the curve (AUC) of Penh against time between 0 and 40 min was calculated for each individual mouse and this figure was used for statistical analysis.

In the long protocol, 22 h after each challenge, reactivity to methacholine was assessed by whole body plethysmography. In the short protocol, 1, 2, 3 or 4 days after the last intranasal challenge, reactivity to methacholine was assessed by whole body plethysmography. Briefly, Penh was calculated for each mouse under resting condition (baseline) and after inhalation, during 1 min, of nebulized (PARI TurboBOY, total output 500 mg/min, PARI GmbH, Starnberg, Germany) methacholine (successively 0, 5, 10, 25 and 50 mg/ml). An average Penh over 30 s was calculated during 3 min (six measurements), and the mean of these six values was used for each concentration. For each mouse, Penh was plotted against methacholine concentration (from 0 to 50 mg/ml) and the AUC was calculated.

In additional experiments carried out to validate the findings obtained by whole body plethysmography, airway responses were measured using a forced oscillation technique (FlexiVent, SCIREQ, Montreal, Canada). This was done in a separate group of mice that received 1, 3 or 6 intranasal instillations in the long model and in all separate groups of the short-protocol model. Lung resistance (R_L) was assessed after increasing exposures to methacholine. Mice were anesthetized with an intraperitoneal injection of pentobarbital (70 mg/kg). The trachea was exposed and an 18-gauge metal needle was inserted into the trachea. Mice were connected to a computer-controlled small animal ventilator and quasi-sinusoidally ventilated with a tidal volume of 10 ml/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H₂O to achieve a mean lung volume close to that during spontaneous breathing. After measurement of a baseline, each mouse was challenged with methacholine aerosol, generated with an in-line nebulizer and administered directly through the ventilator for 5 s, with increasing concentrations (0, 0.3125, 0.625, 1.25, 2.5, 5 and 10 mg/ml). Lung resistance (R_L) was measured using a “snapshot” protocol each 20 s for 2 min. The mean of these six values was used for each methacholine concentration, unless the COD (coefficient of determination) of a measurement was smaller than 0.95. For each mouse, R_L was plotted against methacholine concentration (from 0 to 10 mg/ml) and the AUC was calculated.

2.5. Pulmonary inflammation (bronchoalveolar lavage)

In the long-exposure protocol, 24 h after the last intranasal instillation mice were deeply anesthetized by an intraperitoneal injection of pentobarbital (90 mg/kg body weight), while in the short-exposure protocol, the mice were deeply anesthetized by an intraperitoneal injection of pentobarbital (90 mg/kg body weight) 1, 2, 3 or 4 days after the last intranasal instillation. Blood was first sampled from the retro-orbital plexus, and the mice were then killed by section of the abdominal vessels. Serum was obtained by centrifugation of whole blood at room temperature (14,000 × g, 10 min). Serum samples were stored at –80 °C awaiting further analysis. The lungs were lavaged, *in situ*, three times with 0.7 ml sterile saline (0.9% NaCl), and the recovered fluid was pooled. Cells were counted using a Bürker hemocytometer (total cells) and the bronchoalveolar lavage (BAL) fluid was centrifuged (1000 × g, 10 min). The supernatant was frozen (–80 °C) until further analyses. For differential cell counts, 250 µl of the resuspended cells (100,000 cells/ml) were spun (1400 × g, 6 min) (Cytospin 3, Shandon, TechGen, Zellik, Belgium) onto microscope slides, air-dried and stained (Diff-Quik® method, Medical Diagnostics, Düringen, Germany). For each sample, 200 cells were counted for the number of macrophages, eosinophils, neutrophils and lymphocytes.

Levels of tumor necrosis factor- α (TNF- α), and macrophage inflammatory protein-two (MIP-2) were measured in undiluted BAL fluid by a sandwich enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (TNF- α : Biosource, Nivelles, Belgium; MIP-2: R&D Systems, Abingdon, UK). Lower limits of detection were 3.0 pg/ml and 1.5 pg/ml for TNF- α and MIP-2, respectively. These data are presented in the on-line supplement.

2.6. Lymph node cells (LNC)

Retro-auricular and superficial cervical lymph nodes were also obtained from the same mice. The lymph nodes from 2 to 3 mice were pooled and kept on ice in RPMI-1640 (Invitrogen, Merelbeke, Belgium). Cell suspensions were obtained by pressing the lymph nodes through a cell strainer (100 µm) (BD Bioscience, Erembodegem, Belgium) and rinsing with 10 ml tissue culture medium (RPMI-1640). Cells were counted using a Bürker hemocytometer. Lymphocytes were washed two times and suspended (10⁷ cells/ml) in complete tissue culture medium (RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 10 mg/ml streptomycin, 100 IU/ml penicillin, 1 mM sodium pyruvate, non-essential amino acids).

Five hundred thousand cells were stained with anti-CD3 (APC), anti-CD4 (PE) and anti-CD8 (PerCP-Cy5.5), or received a single staining with anti-CD19 (PE) labeled antibodies, according to standard procedures, and with control samples

being labeled with isotype match control antibodies (BD Biosciences, Erembodegem, Belgium). Flow cytometry (Facsarray, BD Biosciences, Erembodegem, Belgium) was performed using at least 10⁵ cells. These data are presented in the on-line supplement.

Cells were also seeded into 48-well culture plates at a density of 10⁶ cells/ml and incubated in complete RPMI-1640 medium for 18 h without or with 2.5 µg/ml of concanavaline A (Con A) (Sigma–Aldrich, Bornem, Belgium). Cells were then centrifuged (1000 × g, 10 min) and supernatants were stored at –80 °C. Concentrations of interleukin-4 (IL-4) and interferon- γ (IFN- γ) were measured in undiluted supernatant by standard ELISA techniques, according to the manufacturer's instructions (Biosource, Nivelles, Belgium). Lower detection limits were 5 pg/ml and 1 pg/ml for IL-4 and IFN- γ , respectively.

The lymph nodes of 2–3 mice were pooled per experiment. For each group and each time point, 4–6 experiments were performed to obtain a substantial *n*-value for analysis.

2.7. Total serum IgE, IgG₁ and IgG_{2a}

The OptEIA™ Mouse IgE and IgG_{2a} set from Pharmingen (BD Biosciences, Erembodegem, Belgium) was used to measure total serum IgE (diluted 1/70) and total serum IgG_{2a} (diluted 1/10,000). Measurements were performed according to the manufacturer's instructions. For measurement of total serum IgG₁ (diluted 1/250,000), plates were coated using purified anti-mouse IgG₁, a standard was created using purified mouse IgG₁ and the further measurements were performed according to manufacturer's (BD Pharmingen, Erembodegem, Belgium) instructions with the use of biotinylated anti-mouse IgG₁ and avidin horseradish peroxidase conjugate.

2.8. Data analysis

Almost all data are shown as medians and interquartile range (IQR) and were analyzed using the non-parametric Mann–Whitney *U*-test with a 95% confidence interval (Graphpad Prism 4.01, Graphpad Software Inc., San Diego, USA). Lymphocyte subpopulation data were normally distributed and therefore shown as mean and standard deviation (S.D.). These data were analyzed using a student's *t*-test with a 95% confidence interval. A level of *p* < 0.05 (two-tailed) was considered significant.

3. Results

3.1. Ventilatory function

Fig. 2A shows the magnitude of the early ventilatory (Penh) response immediately after each last challenge with TDI or vehicle in the long-treatment protocol. A significant increase in the AUC of Penh was found in the TDI-treated animals after 1 and 2 challenges, but this was no longer present with increasing numbers of challenges. Fig. 2B shows the early ventilatory response immediately after the last (6th) intranasal instillation in the short-treatment protocol. TDI-treated mice show a slight nonsignificant increase in Penh after the 6th intranasal instillation of TDI, compared with the control group.

Fig. 3A shows the responsiveness to increasing concentrations of methacholine on the day after challenge in the long-treatment protocol. When measured with the whole body plethysmograph (AUC Penh, Fig. 3A) the methacholine reactivity shows a more than threefold increase in the TDI-treated group compared to the control group, up to 3 intranasal challenges. The non-specific airway responsiveness, as measured by the resistance of the lung (AUC R_L , Fig. 3C), differed significantly between the vehicle and TDI-treated group only after 1 and 3 intranasal instillations. The right panels of Fig. 3 show increased methacholine responsiveness (Fig. 3B = Penh and Fig. 3D = R_L) up to 4 days after the last intranasal instillation in TDI sensitized and challenged mice (short-treatment protocol). A decrease in responsiveness can be seen in both the TDI and control groups, with increasing time between the last challenge and the methacholine provocation.

3.2. Pulmonary inflammation (BAL)

Fig. 4A, C and E shows the total and differential cell count in the BAL fluid 24 h after each intranasal challenge in the long-treatment protocol. The total cell count (Fig. 4A) was significantly increased until 4r challenges in the TDI-treated group compared to the con-

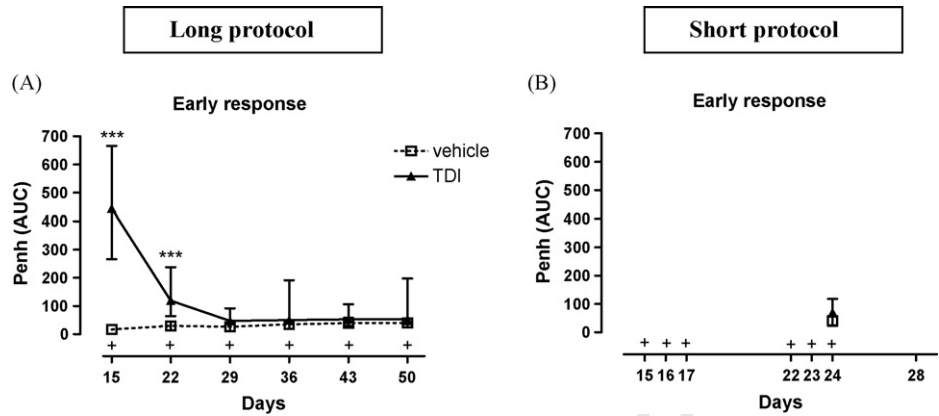


Fig. 2. Early response (Penh) after the intranasal challenge. Early response, expressed as Penh (enhanced pause), was measured by whole body plethysmography. The mice were dermally treated with vehicle (acetone–olive oil) or 0.3% TDI and 1–6 times intranasally challenged (marked with + symbol) with the vehicle or 0.1% TDI. *Long-challenge protocol* (A). Six intranasal challenges were performed with 1-week interval. Graph shows the mean and S.D. of the area under the curve (AUC) of Penh against time between 0 and 40 min in mice, which received 1, 2, 3, 4, 5 or 6 intranasal challenges with vehicle or TDI. *Short-challenge protocol* (B). Six intranasal challenges were performed over a period of 10 days. Graph shows the median and IQR of the AUC of Penh against time between 0 and 40 min after the last (6th) instillation of vehicle or TDI. $n = 9–10$ per group and per time point. *** $p < 0.001$ compared to the control group.

tol group. Mice sensitized and challenged with TDI had significant increases in the percentage of neutrophils (Fig. 4C). An increase of 15–20% neutrophils was seen after 1 and 2 challenges, but with 3 or more challenges the proportion of neutrophils decreased, to become similar to the control group. The percent of eosinophils was only significantly increased after 3 intranasal challenges (Fig. 4E). With 4 or more intranasal instillations, no eosinophils were found anymore.

The right panels (Fig. 4B, D and F) show the BAL cell count results of the short-treatment protocol. There were no statistical differences between TDI and vehicle-treated mice in total cell count (Fig. 4B). Nevertheless, a significant increase in both percent neutrophils (Fig. 4D) and percent eosinophils (Fig. 4F) was found in TDI-treated mice, compared to the control group. This significant difference disappeared 3 days after the last intranasal instillation.

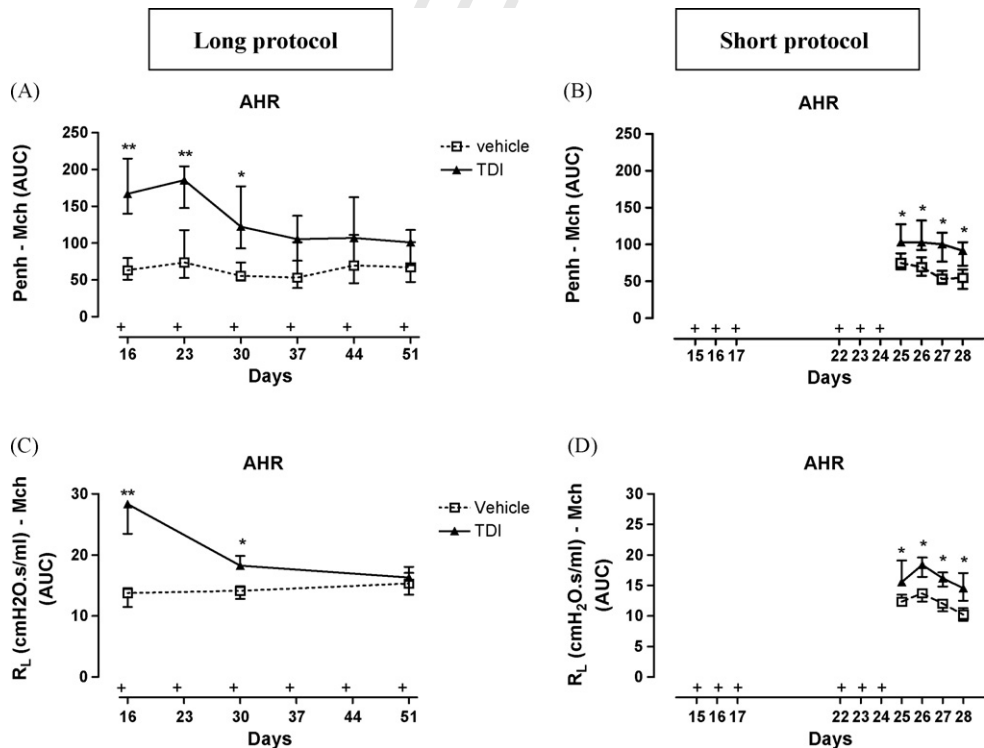


Fig. 3. Methacholine (Mch) responsiveness 22 h after an intranasal challenge. Airway reactivity, expressed as Penh or R_L , was measured by whole body plethysmography or Flexivent, respectively. The mice were dermally treated with vehicle (acetone–olive oil) or 0.3% TDI and 1–6 times intranasally challenged (marked with + symbol) with the vehicle or 0.1% TDI. *Long-challenge protocol*. Methacholine provocation was performed 1 day after each challenge. *Short-challenge protocol*. Methacholine provocation was performed 1, 2, 3 or 4 days after the last intranasal challenge. (A and C) Show the median and IQR of the AUC of Penh against methacholine concentrations of 0–50 mg/ml. (B and D) Show the median and IQR of the AUC of R_L (resistance) against methacholine concentrations of 0–10 mg/ml. The mice in figures A and C ($n = 4–5$) are different from those in figures B and D ($n = 5$). * $p < 0.05$, ** $p < 0.01$ compared with the control group.

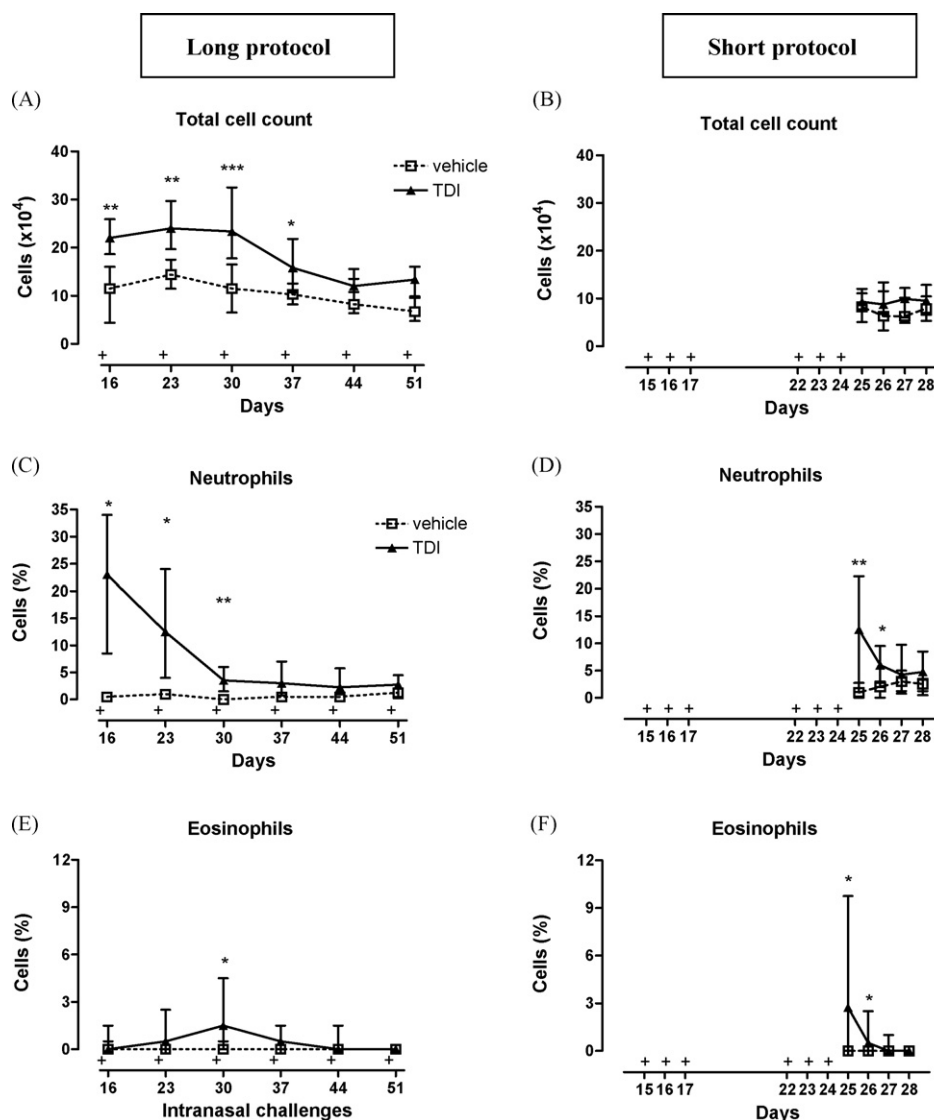


Fig. 4. Total cell count and percent neutrophils and eosinophils in BAL. Cells were obtained by BAL of both lungs. The mice were dermally treated with vehicle (acetone–olive oil) or 0.3% TDI and 1–6 times intranasally challenged (marked with + symbol) with the vehicle or 0.1% TDI. *Long-challenge protocol.* BAL was collected 1 day after each challenge. *Short-challenge protocol.* BAL was collected 1, 2, 3 or 4 days after the last intranasal challenge. (A and B) show the median and IQR of the BAL total cell count. (C and D) show the median and IQR of the percentage neutrophils in BAL. (E and F) show the median and IQR of the percentage eosinophils in BAL. $n = 9$ –10 mice per group and per time point. * $p < 0.05$, ** $p < 0.01$ compared with the control group.

3.3. Lymphocyte cytokine analysis

Fig. 5 (left panel) shows the *in vitro* release of IL-4 and IFN- γ in the supernatants of the auricular and cervical lymph node cells cultured in the presence of Con A from mice treated in the long protocol. Only IL-4 concentrations were elevated in the auricular as well as in the cervical lymph nodes of TDI-treated mice, after Con A stimulation. With increasing numbers of intranasal instillations, the secretion of IL-4 by auricular lymphocytes decreased (Fig. 5A), but it increased for cervical lymphocytes (Fig. 5C). The levels of IFN- γ (Fig. 5E and G), secreted by lymphocytes of TDI-treated mice, were increased, but this was only statistically different from the controls until 3 intranasal challenges for the auricular lymphocytes (Fig. 5E) and only after 6 intranasal challenges for the cervical lymphocytes (Fig. 5G).

The right panels of Fig. 5 show the results of the supernatant of auricular and cervical lymph nodes of mice treated in the short protocol. TDI-treated mice showed up to 4 days after the last intranasal instillation with TDI a significant increase of IL-4 in the supernatant

of auricular and cervical lymphocytes, after Con A stimulation, compared to the control group (Fig. 5B and D). Secretion of IFN- γ , in the supernatant of auricular as well as cervical lymphocytes, was significantly increased in TDI-treated mice until 2 days after the last intranasal instillation, compared to the controls (Fig. 5F and H).

3.4. Total serum IgE, IgG₁ and IgG_{2a}

The left panels of Fig. 6 show the concentrations of total serum IgE, IgG₁ and IgG_{2a} in mice treated in the long protocol. The TDI-treated mice showed significantly higher levels of total serum IgE (Fig. 6A) compared with the vehicle treated mice. No significant differences were found in total serum IgG₁ (Fig. 6C) and IgG_{2a} (Fig. 6E). Levels of IgE and IgG_{2a} increased with time and number of intranasal instillations in both the vehicle and TDI-treated mice. The right panels of Fig. 6 show the total serum IgE, IgG₁ and IgG_{2a} in mice treated in the short protocol. Fig. 6B shows a significant nine-fold increase of total serum IgE in TDI-treated mice compared to the

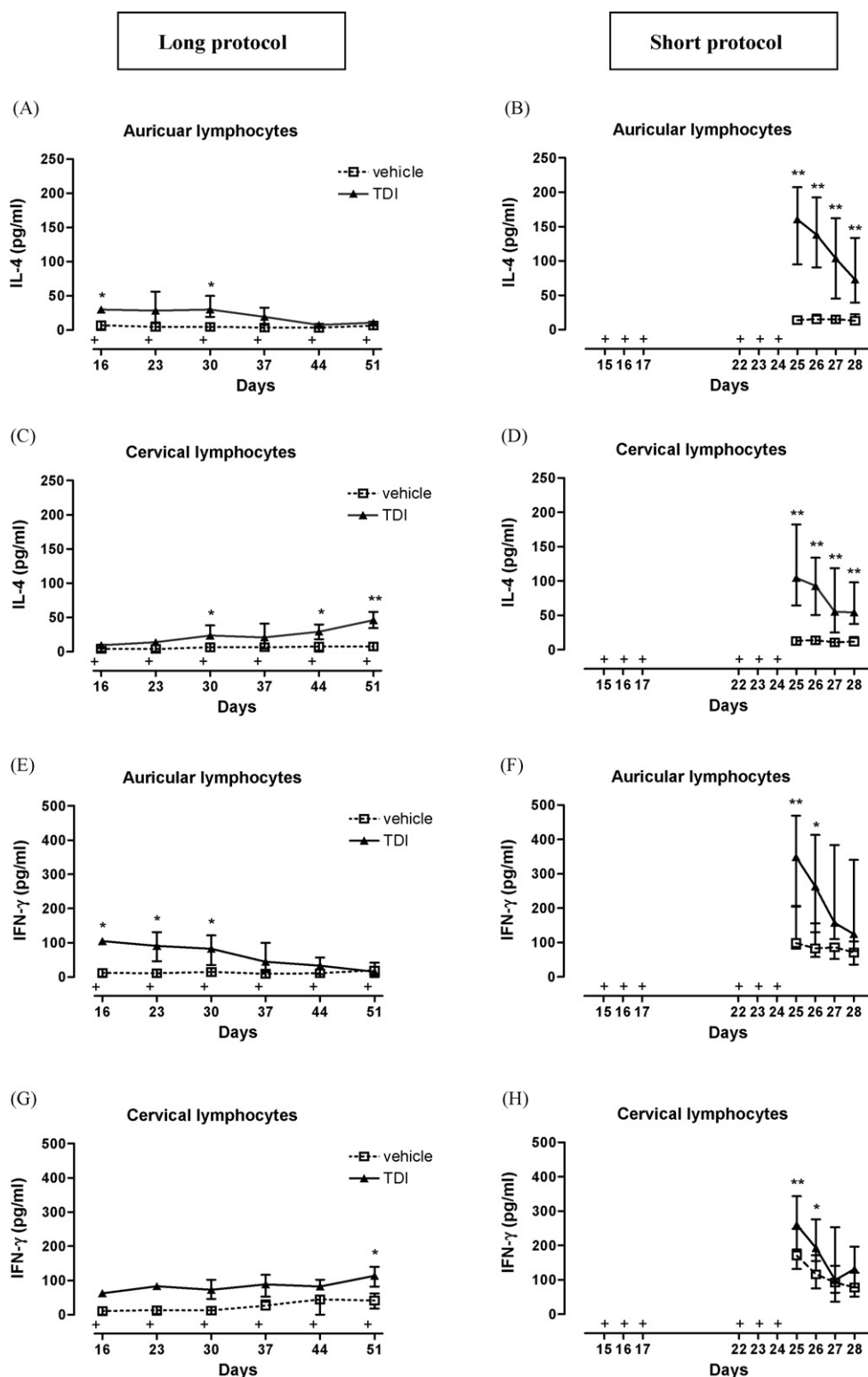


Fig. 5. IL-4 and IFN- γ secretion in auricular and cervical lymphocyte supernatant. The lymph nodes of 2 to 3 mice were pooled, and the obtained lymph node cells were cultured (18 h) with concanavaline A (Con A, 2.5 μ g/ml) stimulation. The mice were dermally treated with vehicle (acetone–olive oil) or 0.3% TDI and 1–6 times intranasally challenged (marked with + symbol) with the vehicle or 0.1% TDI. *Long-challenge protocol.* Lymph nodes were collected 1 day after each challenge. *Short-challenge protocol.* (A and B) is the median and IQR level of IL-4 in supernatant of auricular lymphocytes. (C and D) is the median and IQR level of IL-4 in supernatant of cervical lymphocytes. (E and F) is the median and IQR level of IFN- γ in supernatant of auricular lymphocytes. (G and H) is the median and IQR level of IFN- γ in supernatant of cervical lymphocytes. $n=4$ –6 experiments per group and per time point. * $p < 0.05$, ** $p < 0.01$ compared with the control group.

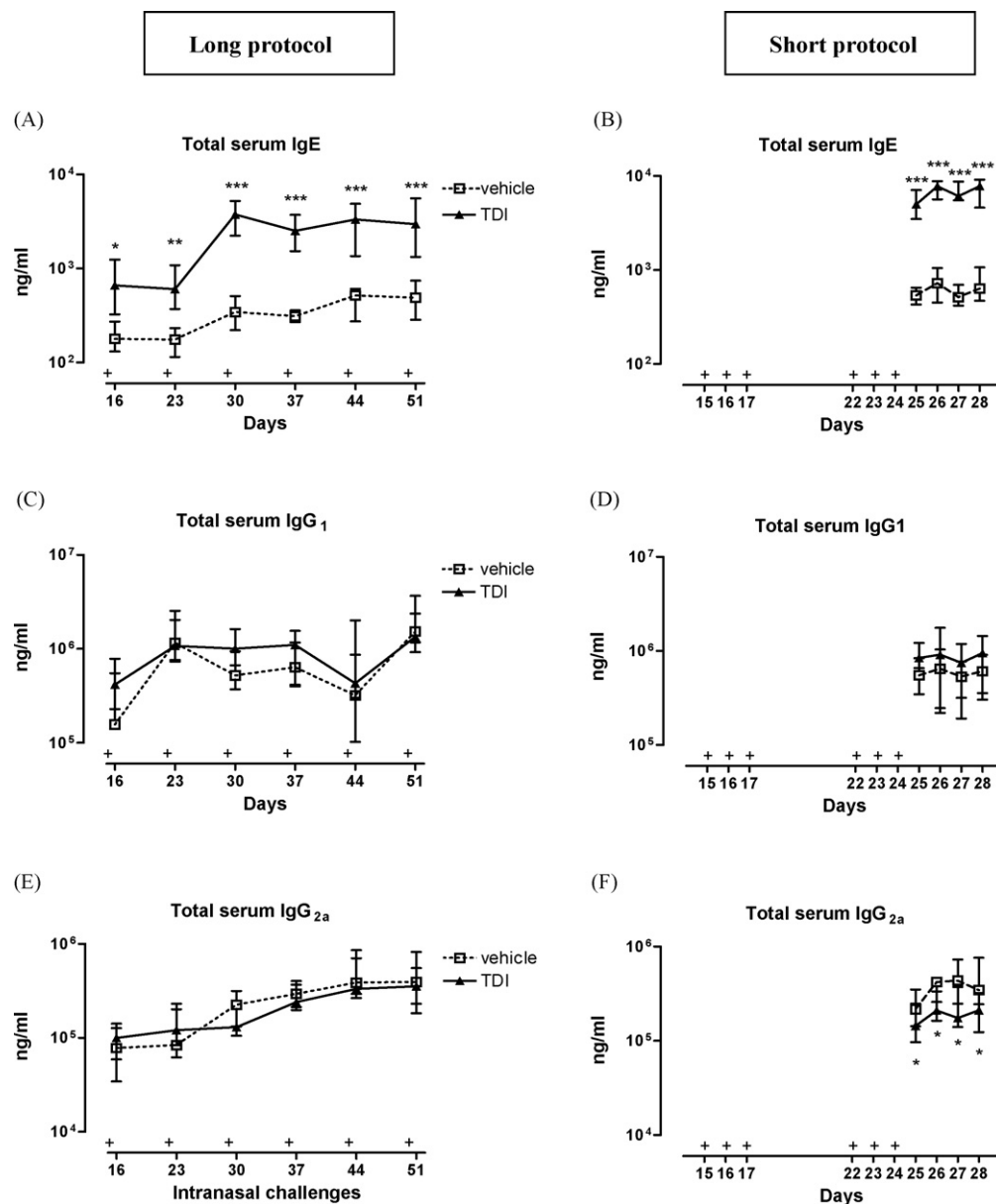


Fig. 6. Total serum IgE, IgG₁ and IgG_{2a} levels. Total serum immunoglobulin levels were measured using standard ELISA techniques. The mice were dermally treated with vehicle (acetone–olive oil) or 0.3% TDI and 1–6 times intranasally challenged (marked with + symbol) with the vehicle or 0.1% TDI. Long-challenge protocol. Blood was collected 1 day after each challenge. Short-challenge protocol. Blood was collected 1, 2, 3 or 4 days after the last intranasal challenge. (A and B) show the median and IQR of total serum IgE levels. (C and D) show the median and IQR of total serum IgG₁ levels. (E and F) show the median and IQR of total serum IgG_{2a} levels. Note that the scale is different in the graphs. $n = 9$ –10 per group and per time point. * $p < 0.05$, *** $p < 0.001$ compared with the control group.

control group at all time points of autopsy. Total serum IgG_{2a} levels (Fig. 6F) were significantly decreased in TDI-treated mice. We found no significant differences between total serum IgG₁ concentrations of TDI- and vehicle-treated mice (Fig. 6D).

4. Discussion

Previously we described a mouse model where the mice were intranasally challenged once after dermal sensitization (Tarkowski et al., 2007; Vanoirbeek et al., 2006a, 2008). The objective of the present study was to create a more robust response after challenge with TDI. Therefore, we gave multiple intranasal challenges, up to 6, with 1-week recovery between each challenge. Contrary to our expectation, both the physiologic and inflammatory responses to TDI diminished with increasing challenges. This occurred in spite of persisting signs of lymphocyte activation and humoral antibodies.

Following these results, we introduced a shorter challenge protocol, in which we intranasally challenged the mice six times over a period of 10 days. We also tested whether the time point of assessment after the last challenge would improve our results.

As already shown previously, our measurements indicate the existence of a persistent systemic immune response following dermal sensitization and intranasal challenges (Tarkowski et al., 2007; Vanoirbeek et al., 2004, 2008). The increase in concentration of total serum IgE evolves to a plateau and remains constant until 6 intranasal challenges (both in the long and short protocol). This has also been described by other research groups (Ban et al., 2006). Although, the importance of IgE in diisocyanate asthma has not yet been proven (Jones et al., 2006), we can only conclude that we have a stimulation of the Th2-pathway [increased % of B-cells (see online supplement) and total serum IgE] in this experimental setup. Nevertheless, this phenomenon does not appear to be linked to the

development of the asthma-like phenotype in mice. Total serum IgG₁ does not change in these experiments. This was surprising, since IgG₁ has been suggested as a marker of diisocyanate exposure, more than an indicator of sensitization in human diisocyanate asthma (Park et al., 2002).

In the short-treatment protocol, we describe for the first time a decrease of total serum IgG_{2a} in this model, which suggests an inhibition of a Th1 response. It has already been shown that peptides containing the dominant T-cell epitope of Ole e 1 (main allergen of olive pollen) can suppress serum specific IgE, IgG₁ and IgG_{2a} (Marazuela et al., 2008). Besides this polyclonal immunoglobulin inhibition, it has also been shown, that OVA-induced oral tolerance can cause a decrease in IgG_{2a} levels (Mito et al., 2006). To our knowledge, a decrease in total serum IgG_{2a} after exposure to a chemical antigen has never been shown. Nevertheless, our IgG_{2a} data are somewhat in contrast with our own data from the local draining lymph nodes. Con A-stimulated lymphocytes from both auricular and cervical lymph nodes showed a pronounced Th2 response (elevated IL-4 secretion), but also signs of a significant Th1 response (elevated IFN- γ secretion) 1 and 2 days after the last intranasal instillation. However, with increasing time after the last challenge, both the Th1 and Th2 response disappears. While Th1 cytokines normally are considered to have a negative influence on allergic Th2 responses, more and more data show that there is a cooperative action between Th1 and Th2 cytokines in the pathogenesis of chemical-induced asthma in mice (Johnson et al., 2004; Matheson et al., 2005a). Moreover, studies in humans have shown that IL-13-induced lung inflammation is positively influenced by IFN- γ (Ford et al., 2001) and that the number of IFN- γ producing cells found in asthmatic lungs correlates with asthma severity and AHR (van Rijt and Lambrecht, 2001).

Surprisingly, the early and late ventilatory responses declined with increasing challenges (long protocol) and with increasing time after the last challenge (short protocol). Along with this decline, the neutrophil chemoattractant MIP-2 (see on-line supplement) and the number of neutrophils also decreased rapidly. With the decrease in percentage neutrophils we expected to find an increase in eosinophils. After 2 or 3 intranasal instillations in the long protocol, we did find a limited increase in eosinophils in BAL, but these disappeared again with increasing TDI-challenges. In the shorter challenge protocol, we tested whether the time point of assessment after the last challenge would improve our results. We know from our single intranasal instillation protocol, that neutrophils appear in BAL, 1 h after the challenge (Tarkowski et al., 2007). Since we did not find eosinophils in BAL at any time between 1 and 24 h after a single instillation, we thought that these cells perhaps would appear later than neutrophils in BAL. However, in the short-protocol experiments we observed a small number of eosinophils 1 and 2 days after intranasal challenge. Along with the eosinophils, the neutrophils, also rapidly decreased after the last intranasal challenge, indicating that the mice easily recuperate from these intranasal challenges. This rapid decrease of the lung inflammation occurred in parallel with the decreased airway hyper-reactivity in the TDI-treated mice. Overall, this decrease in response was unexpected, since in literature several multiple exposure protocols are described which yield better results than single exposure assays (Ban et al., 2006; Matheson et al., 2005b; Pauluhn, 2008a,b). Several valid explanations can be found in order these apparent inconsistencies. Dose–response relationships are, without doubt, important in these models. The more persistent phenotype of allergic lung inflammation and remodeling requires chronic repeated challenges to haptens, rather than all-or-nothing, single sensitization excursions (Pauluhn, 2008a). The mode of application (dermal sensitization and inhalation vs. intranasal challenge) and dosing (high vs. low dose for sensitization and challenge) will determine the outcome of the experiments (Matheson et al., 2005b; Pauluhn, 2003, 2008a). In

our experiments, multiple intranasal challenges were probably not the best way to challenge the mice repeatedly, because the nasal epithelial is targeted too much. Nevertheless, in order to replace costly and difficult aerosol exposure experiments, intranasal challenge has been proven physiologically adequate (Southam et al., 2002). Aerosol inhalation challenge, also targets firstly the nose, is a more real-life exposure as indicated by Pauluhn et al. (Pauluhn, 2008a) or intratracheal instillation and oro-pharyngeal aspiration can be an alternative in the future. These techniques bypass the nose and targets the lung more directly (Ban et al., 2006).

In our results, we interpret this decrease in airway and inflammatory response as evidence for the development of “tolerance” by giving multiple intranasal challenges. Van Hove et al. and Tournoy et al. found that prolonged exposures to ovalbumin can result in a complete waning of inflammation and they explained this by an “exhaustion” of the immune system. This immune tolerance was attributed to an inhibition of dendritic cell maturation and of the co-stimulatory molecules on dendritic cells and T-lymphocytes (Tournoy et al., 2006a; Van Hove et al., 2007). Schramm et al. found that tolerance was a local phenomenon, inhibiting airway inflammation but not systemic IgE responses to antigen exposure, which corresponds to our findings (Schramm et al., 2004). Moreover, from unpublished data we have indications that the numbers of CD25⁺ Tregs in the draining lymph nodes are increased in TDI-sensitized and challenged mice, which could be the reason for the diminished asthmatic response in these mice. The down regulation of the asthmatic phenotype by Tregs, via IL-10, has been shown in both human and mouse asthma studies (Bacchetta et al., 2007; Hammad et al., 2007; Seroogy and Gern, 2005).

Both the long- and short-multiple intranasal challenge protocols with a known chemical sensitizer yielded a blunting of the airway responses, while the mice obviously remained allergic, as evidenced by several parameters (IgE and IL-4). Therefore, we conclude that these multiple intranasal challenges protocols offer a new model for research into the mechanisms of tolerance and open perspectives for identifying the cells and pathways that play a key-role. Candidates for these are B-cells, Treg cells (CD25⁺-FOXP3), dendritic cell subtypes and cytokines like IL-10 and TGF- β (Akbari et al., 2001; Ostroukhova et al., 2004; Tournoy et al., 2006b; Tsitoura et al., 2002). It could well be that people with isocyanate-induced occupational asthma have a defect in the mechanisms that lead to tolerance. Better insights in these mechanisms can lead to new therapeutic interventions for allergic asthma induced by either high or low-molecular weight agents.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2008.12.020.

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On-line supplement: Multiple challenges in a mouse model of chemical-induced asthma lead to tolerance: ventilatory and inflammatory responses are blunted, immunologic humoral responses are not.

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Results

Pulmonary inflammation (BAL)

Figure 1A shows the changes in MIP-2 concentrations in the BAL fluid of mice treated with the long protocol. A minor increase in MIP-2 concentration was observed in the TDI treated group, but this was only significant after 1 and 2 intranasal challenges. No differences in TNF- α concentrations were found between the TDI and vehicle treated groups (data not shown).

Figure 1B shows the changes in MIP-2 concentration in BAL fluid of mice treated with the short protocol. Compared to the control group, TDI-treated mice showed an increase in MIP-2 concentration until 2 days after the last intranasal instillation. TNF- α concentration was not significantly different between control and TDI-treated mice at the different time points (data not shown).

Lymphocyte subpopulation analysis

Figure E2 (left panel) shows the different subpopulations of lymphocytes in the auricular lymph nodes in the long treatment protocol. A decrease in the proportion of CD3⁺CD4⁺ (T-helper lymphocytes) (Fig. 2A) and CD3⁺CD8⁺ (T-cytotoxic lymphocytes) (Fig. 2C) lymphocytes was observed in the auricular lymph nodes. After 4 challenges the subpopulations in the lymph nodes returned to the proportions found in the lymph nodes of vehicle treated mice. A significant increase in the proportion of CD19⁺ lymphocytes (B-lymphocytes) (Fig. 2E) was found in auricular lymph nodes in the TDI treated group, compared to the control group.

Figure E2 (right panel) shows the different lymphocyte subpopulations in the auricular lymph nodes in the short treatment protocol. A significant decrease in the proportions of CD3⁺CD4⁺ cells (Fig. 2B) and CD3⁺CD8⁺ cells (Fig. 2D) and an increase in the proportion of CD19⁺ cells (Fig. 2F) was found in the auricular lymph nodes of TDI-treated mice up to 4 days after the last intranasal instillation.

Figure E3 (left panel) shows the different subpopulations of lymphocytes in the cervical lymph nodes in the long treatment protocol. A decrease in the proportion of CD3⁺CD4⁺ (Th lymphocytes) (Fig. 3A) and CD3⁺CD8⁺ (Tc lymphocytes) (Fig. 3C) lymphocytes was observed in the cervical lymph nodes. After 3 challenges the subpopulations in the lymph nodes returned to the proportions found in the lymph nodes of vehicle treated mice. A significant increase in the proportion of CD19⁺ lymphocytes (B-lymphocytes) (Fig. 3E) was found in the cervical lymph nodes in the TDI treated group, compared to the control group.

Figure E3 (right panel) shows the different lymphocyte subpopulations in the cervical lymph nodes in the short treatment protocol. A significant decrease in the proportions of CD3⁺CD4⁺ cells (Fig. 3B) and CD3⁺CD8⁺ cells (Fig. 3D) and an increase in the proportion of CD19⁺ cells (Fig. 3F) was found in the cervical lymph nodes of TDI-treated mice. The proportions of the different lymphocyte subpopulations in the cervical lymph nodes showed a similar trend as the auricular lymph nodes. However, in the cervical lymph nodes, no differences in subpopulations were found 4 days after the last challenge (Fig. 3 B, D and F).

Legend

Figure 1: **MIP-2 in BAL after the last intranasal instillation of TDI or vehicle.**

MIP-2 concentrations in BAL were measured by ELISA. The mice were dermally treated with vehicle (acetone-olive oil) or 0.3% TDI and 1 to 6 times intranasally challenged (marked with + symbol) with the vehicle or 0.1% TDI. **Long challenge protocol (A) (see Fig. 1a).** BAL was collected one day after each challenge. **Short challenge protocol (B) (see Fig. 1b).** BAL was collected 1, 2, 3 and 4 days after the last challenge. The graphs show median and IQR of the BAL MIP-2 concentration. n = 9-10 mice per group and per time point. * p<0.05 compared with the control group.

Figure 2: **T-helper (CD3⁺CD4⁺), T-cytotoxic (CD3⁺CD8⁺) and B-lymphocyte (CD19⁺) subpopulations in auricular lymph nodes.**

The mice were dermally treated with vehicle (acetone-olive oil) or 0.3% TDI and 1 to 6 times intranasally challenged (marked with + symbol) with the vehicle or 0.1% TDI. The auricular lymph nodes of 2 to 3 mice were pooled, and the obtained lymph node cells were stained with anti-CD3, anti-CD4 (**A and B**) and anti-CD8 (**C and D**), or received a single staining with anti-CD19 (**E and F**) labeled antibodies. Flow cytometry was performed using at least 10⁴ cells. **Long challenge protocol (see Fig. 1a).** Lymph nodes were collected one day after each challenge. **Short challenge protocol (see Fig. 1b).** Lymph nodes were collected 1, 2, 3 or 4 days after the last intranasal challenge. n = 4-6 experiments per group and per time point. All graphs show mean and SD. * p<0.05, ** p<0.01, ***p<0.001 compared with the control group.

Figure 3: T-helper ($CD3^+CD4^+$), T-cytotoxic ($CD3^+CD8^+$) and B-lymphocyte ($CD19^+$) subpopulations in cervical lymph nodes

The mice were dermally treated with vehicle (acetone-olive oil) or 0.3% TDI and 1 to 6 times intranasally challenged (marked with + symbol) with the vehicle or 0.1% TDI. The cervical lymph nodes of 2 to 3 mice were pooled, and the obtained lymph node cells were stained with anti-CD3, anti-CD4 (**A and B**) and anti-CD8 (**C and D**), or received a single staining with anti-CD19 (**E and F**) labeled antibodies. Flow cytometry was performed using at least 10^4 cells. **Long challenge protocol (see Fig. 1a)**. Lymph nodes were collected one day after each challenge. **Short challenge protocol (see Fig. 1b)**. Lymph nodes were collected 1, 2, 3 or 4 days after the last intranasal challenge. n = 4-6 experiments per group and per time point. All graphs show mean and SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the control group.

Figures

Figure 1

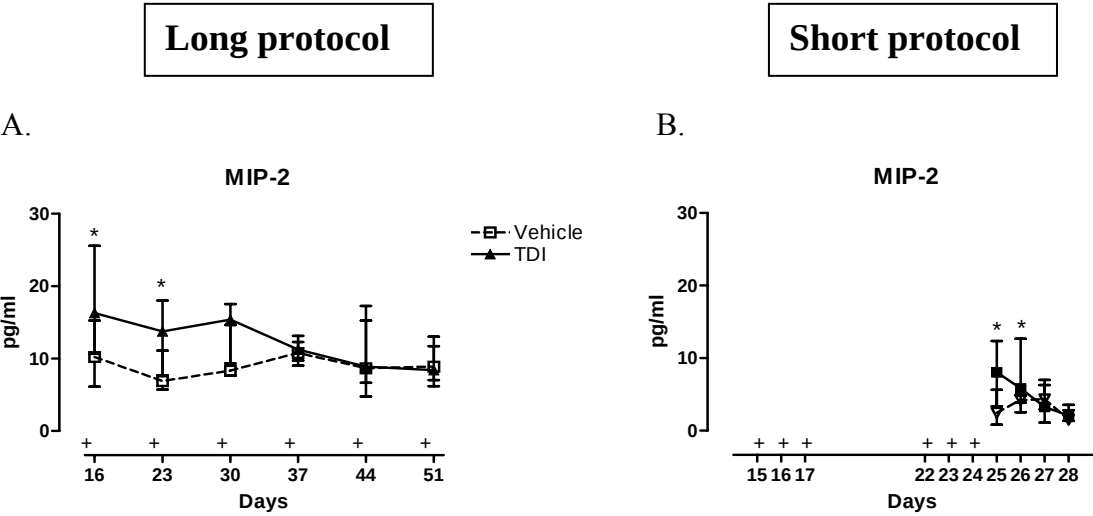


Figure 2

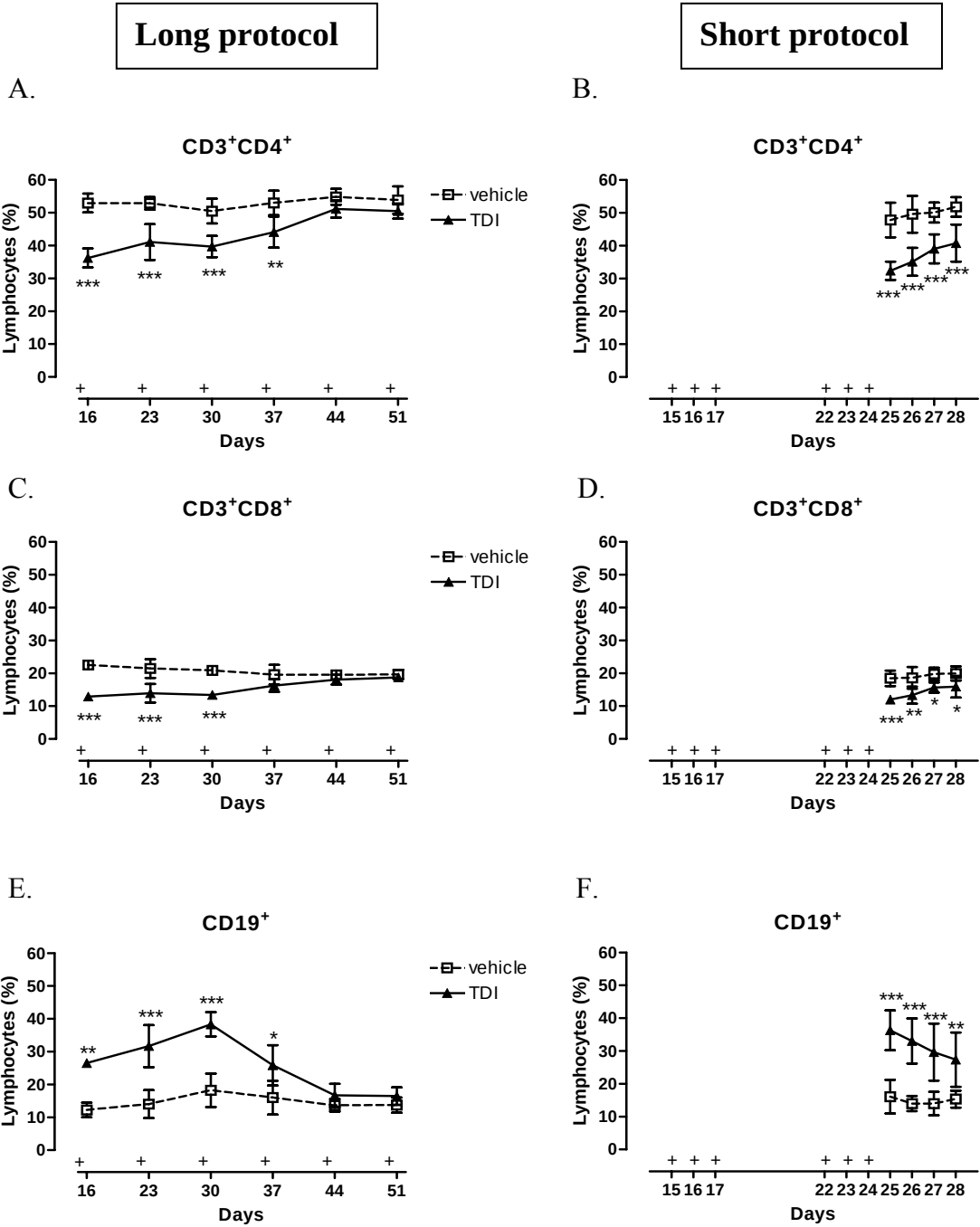


Figure 3

