

How long do the systemic and ventilatory responses to toluene diisocyanate persist in dermally sensitized mice?

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Background: Years after removal from exposure, workers with occupational asthma still show respiratory symptoms and airway hyperresponsiveness on re-exposure to the offending agent.

Objective: We investigated the persistence of the respiratory responsiveness to toluene diisocyanate (TDI) in a mouse model. **Methods:** BALB/C mice received dermal applications of TDI on days 1 and 8, and a single intranasal instillation of TDI on day 10, 15, 20, 25, 30, 40, 50, 60, or 90. After instillation, early (1 hour) changes in ventilatory function and methacholine responsiveness (22 hours) were assessed. Cell counts and macrophage inflammatory protein 2 were measured in bronchoalveolar lavage. Total serum IgE, IgG₁, and IgG_{2a} were quantified. Lymphocyte subpopulations were assessed in auricular and cervical lymph nodes, and release of IL-4 and IFN- γ by these lymph node cells was measured.

Results: Toluene diisocyanate-treated mice showed immediate ventilatory changes, increased methacholine reactivity, and an influx of neutrophils and macrophage inflammatory protein 2 in bronchoalveolar lavage as long as 50 days after initial treatment. These mice also showed a relative increase in CD19⁺ cells and a decrease in CD4⁺ and CD8⁺ cells in auricular lymph nodes. Increased release of IL-4 and IFN- γ in auricular lymph node cells was observed only until 20 days after sensitization. Total serum IgE, IgG₁, and IgG_{2a} remained significantly elevated in TDI-sensitized mice until 90 days after dermal sensitization.

Conclusion: Ventilatory and lung inflammatory responses decrease with increasing delay between sensitization and challenge, despite persistent humoral signs of sensitization. **Clinical implications:** This study confirms the dissociation between humoral evidence of allergy to TDI and respiratory responsiveness to TDI. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: TDI, dermal sensitization, occupational asthma, murine model, lymphocyte subtypes, IL-4, IFN- γ , MIP-2

Currently there is no cure for immunologically mediated occupational asthma (OA). The most common treatment of OA is definitive cessation of exposure to the offending agent, which is usually achieved in practice by removal of the employee from the workplace. The rationale for advising a complete and definitive cessation of exposure is that immunologic sensitization does not go away and that any contact with the offending agent is therefore likely to cause a recurrence of the asthma. Nevertheless, it is known—mainly from clinical experience with specific bronchial provocation tests—that bronchial responsiveness to an occupational sensitizer decreases with time away from exposure, and in a minority of subjects it may even disappear.¹⁻⁴ On the other hand, even after complete avoidance of exposure, many subjects with occupational asthma remain asthmatic with varying degrees of clinical severity, medication need, and nonspecific bronchial reactivity.⁵⁻⁷ Lemiere et al⁸ suggested that the outcome is more favorable when patients have been unexposed for 5 years or more.

The reasons for the persistence of asthma and bronchial hyperresponsiveness even after cessation of exposure are unclear. Follow-up studies have demonstrated that subjects without improvement have more airway inflammation,⁹ but why this occurs in some subjects and not in others is still obscure. Although clinical studies in patients are extremely valuable to clarify the pathophysiology of OA and its outcome, studies with suitable animal models of OA are needed to disentangle the various processes underlying the initiation and persistence of specific ventilatory responses, bronchial hyperresponsiveness, airway inflammation, and remodeling. This is the general purpose of our experimental research using a mouse model of chemical-induced asthma.

The pathophysiology of OA is still controversial. It can be mediated by immunologic and nonimmunologic mechanisms, and among the former, IgE-dependent and IgE-independent are known to exist.¹⁰ IgE-dependent OA, which is generally caused by biological agents of high molecular weight, and IgE-independent OA, which is generally caused by (reactive) chemicals of low molecular

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Abbreviations used

AHR:	Airway hyperreactivity
AUC:	Area under the curve
BAL:	Bronchoalveolar lavage
ConA:	Concanavalin A
MIP-2:	Macrophage inflammatory protein 2
OA:	Occupational asthma
Penh:	Enhanced pause
R:	Resistance
TDI:	Toluene diisocyanate

weight, such as isocyanates, have similar clinical features.¹¹ It has been demonstrated that T_H2 activation, with increased levels of IL-4 and IL-5, is crucial in OA.¹² However, not only T_H2 but also T_H1 activation with IFN- γ production is important in OA induced by low-molecular-weight agents, notably through involvement of CD8⁺ $\gamma\delta$ T cells.¹³⁻¹⁵

In our previous studies, we developed and partially validated a mouse model of chemical-induced asthma by using toluene diisocyanate (TDI), in which dermal sensitization was followed by intranasal challenge.¹⁶⁻¹⁸ In this study, we examined how long the respiratory responsiveness to TDI would persist after dermal sensitization. Our hypothesis, based on human experience in OA, was that with increasing time between dermal sensitization and intranasal challenge the respiratory response would decrease, though not disappear completely. Therefore, we compared airway responses, lung inflammation, and immune responses induced by a single intranasal TDI challenge administered at variable intervals (between 2 and 82 days) after dermal sensitization to TDI.

METHODS

A complete description of the materials and methods is available in this article's Online Repository at www.jacionline.org.

All experimental procedures were approved by the local Ethical Committee for Animal Experiments.

Toluene diisocyanate was dissolved in the vehicle (acetone olive oil), consisting of a mixture of 2 volumes of acetone and 3 volumes of olive oil.

Male BALB/c mice (6 weeks old) received dermal applications of 20 μ L vehicle or 20 μ L 0.3% TDI (vol/vol) on each ear on days 1 and 8. On day 10, 15, 20, 25, 30, 40, 50, 60, or 90, they received, under light diethyl ether anesthesia, an intranasal instillation of 10 μ L vehicle or 10 μ L 0.1% TDI (vol/vol) in each nostril. In all experiments, treatment with TDI is indicated as 1 and treatment with the vehicle is indicated as 0. All experimental groups are indicated with 3 symbols: the first and second symbols indicate the dermal treatment on days 1 and 8, whereas the third symbol indicates the intranasal instillation. Thus, the 1/1/1 group consists of mice that received dermal applications of TDI on days 1 and 8, and intranasal instillations of TDI on the day of challenge, whereas the 0/0/0 control group consists of mice that received acetone olive oil on all occasions. For the last time point (day 90), we included 2 extra groups: 0/0/1 and 1/1/0. Each group of mice (vehicle or TDI) consisted of 8 to 10 mice at each time point.

On the day of intranasal challenge, the ventilatory function (enhanced pause [Penh]) was measured by unrestrained whole-body plethysmography (EMKA Technologies, Paris, France) as

previously described.¹⁸ Ventilatory function of each mouse was first recorded in resting condition for 5 minutes. Immediately after the intranasal instillation, mice were placed again in the whole-body plethysmograph (0 time point), and their ventilatory parameters were measured for 40 minutes. The area under the curve (AUC) of Penh against time between 0 and 40 minutes was calculated for each individual mouse, and this figure was used for statistical analysis.

One day (22 hours) after intranasal instillation, reactivity to methacholine was assessed as previously described.¹⁸ Briefly, Penh was calculated for each mouse under resting conditions (baseline) and after inhalations, during 1 minute, of nebulized methacholine (successively 0, 5, 10, 25, and 50 mg/mL) in the whole-body plethysmograph. For each mouse, Penh was plotted against methacholine concentration, and the AUC was calculated. In additional experiments, a forced oscillation technique (FlexiVent; SCIREQ, Montreal, Quebec, Canada) to measure methacholine reactivity was used in separate groups of mice at selected time points (22 hours after challenge given 15, 30, 60, or 90 days after initial dermal contact). Briefly, mice were anesthetized, tracheostomized, and ventilated. Resistance (R) was measured for each mouse under baseline conditions (baseline), and after inhalation, during 5 seconds, of increasing concentrations of nebulized methacholine (successively 0, 0.625, 1.25, 2.5, 5, and 10 mg/mL). For each mouse, R was plotted against methacholine concentration, and the AUC was calculated.

After the methacholine test (ie, 24 hours after intranasal instillation), the mice were deeply anesthetized by intraperitoneal injection of pentobarbital. Blood was first sampled from the retro-orbital plexus for measuring total serum IgE, IgG₁, and IgG_{2a} using a standard ELISA assay. The mice were then killed by section of the abdominal vessels. The lung was lavaged, and the recovered fluid was pooled. Total cells were counted by using a Bürker hemocytometer, and cells were spun onto microscope slides, air-dried, and stained for enumerating macrophages, eosinophils, neutrophils, and lymphocytes.

Levels of TNF- α and macrophage inflammatory protein 2 (MIP-2) were measured in undiluted bronchoalveolar lavage (BAL) fluid by a sandwich ELISA.

Retroauricular 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. Cell suspensions were obtained by pressing the lymph nodes through a cell strainer (100 μ m). Lymphocytes were washed 3 times and suspended (10⁷ cells/mL) in complete tissue culture medium. Five hundred thousand cells were stained with anti-CD3 (allophycocyanine), anti-CD4 (phycoerythrin), and anti-CD8 (peridinin-chlorophyll cyanine), or received a single staining with anti-CD19 (phycoerythrin)-labeled antibodies, with control samples labeled with isotype-match control antibodies. Flow cytometry was performed by using at least 10⁴ cells. Cells were also seeded into 48-well culture plates and incubated in complete RPMI-1640 medium for 18 hours without or with 2.5 μ g/mL concanavalin A (ConA). Supernatants were stored at -80°C. Concentrations of IL-4 and IFN- γ were measured in undiluted supernatant by a standard ELISA assay.

Data are shown as means with SDs. All data were analyzed by using the nonparametric Mann-Whitney *t* test with a 95% CI (GraphPad Prism 4.01, GraphPad Software Inc, San Diego, Calif). A level of *P* < .05 (2-tailed) was considered significant. Nonparametric Spearman correlation test fitting was performed on the results of the TDI-sensitized and challenged groups of mice by using SAS8.2 software (SAS Institute Inc, Tervuren, Belgium).

RESULTS

Ventilatory function

Fig 1, A, shows the early ventilatory responses immediately after intranasal instillation. Mice that had previously

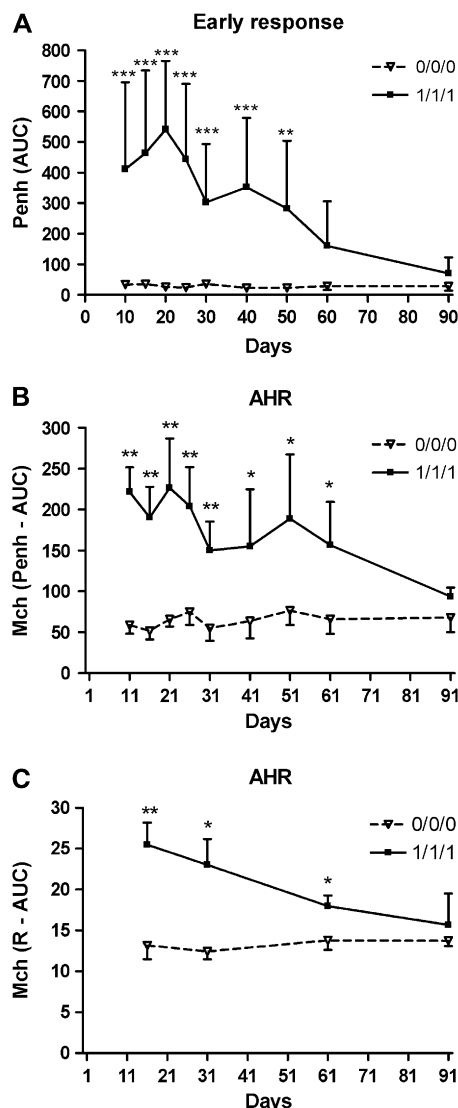


FIG 1. A, Early ventilatory response (Penh) after intranasal instillation with TDI or vehicle. Mean and SD of AUC of Penh against time between 0 and 40 minutes. $n = 8-10$ mice per group, per time point. **B,** Methacholine (Mch) responsiveness (Penh) 1 day after intranasal instillation of TDI or vehicle. Mean and SD of the AUC of Penh against methacholine concentrations of 0 to 50 mg/mL. $n = 4-5$ mice per group, per time point. **C,** Mch responsiveness (R) 1 day after intranasal instillation of TDI or vehicle. Mean and SD of the AUC of R (FlexiVent) against methacholine concentrations of 0 to 10 mg/mL. $n = 4-5$ mice per group, per time point. Experimental groups are identified by 3 symbols. 1 and 0 represent administration of TDI or vehicle (acetone:olive oil) respectively; the first 2 symbols identify the agent (0.3% TDI) applied dermally on day 1 and 8, and the third symbol identifies the agent (0.1% TDI) instilled intranasally. The mice in **C** are different from those in **A** and **B**. * $P < .05$; ** $P < .01$; *** $P < .001$ compared with the control group.

received dermal applications of TDI showed a significant increase in Penh after intranasal instillation of TDI as long as 50 days after initial dermal sensitization, compared with the 0/0/0 control groups. Although there was an apparent peak response on day 20, there was no statistical difference within the TDI-treated group between the

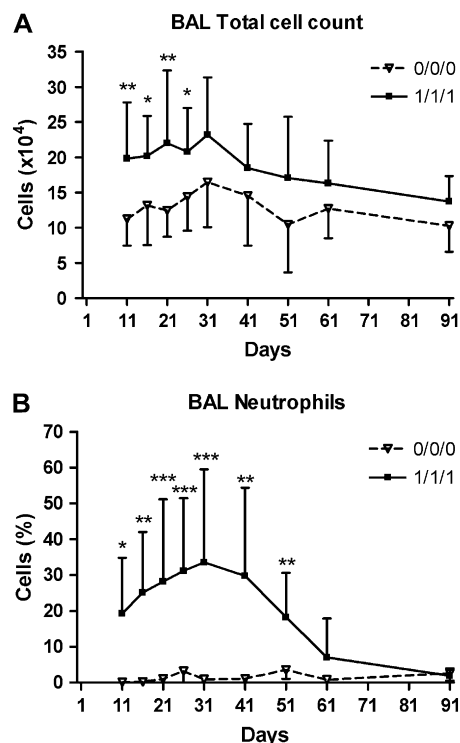


FIG 2. Total cell count and percent neutrophils in BAL 1 day after intranasal instillation of TDI or vehicle. A, Mean and SD of the BAL total cell count. **B,** Mean and SD of the percentage neutrophils in BAL. Experimental groups are as in Fig 1. $n = 8-10$ mice per group. * $P < .05$; ** $P < .01$; *** $P < .001$ compared with the control group.

magnitudes of the early responses at days 10, 15, 20, 25, 30, 40, and 50. The decline in response was linear with time.

Fig 1, **B** and **C**, shows increased methacholine responsiveness (Penh and R) 22 hours after intranasal instillation in mice dermally sensitized and challenged with TDI as long as 61 days after initial dermal sensitization, compared with the 0/0/0 control group. Again, the decline in responsiveness was linear with time.

Pulmonary inflammation (BAL)

Fig 2, **A**, shows an increased total cell count in BAL from mice dermally sensitized and intranasally instilled with TDI (1/1/1), compared with the 0/0/0 control groups. This increase was significant only until day 26. The increase in total cells was attributable to an increase in neutrophils (Fig 2, **B**). In TDI-treated mice (1/1/1), the increase in BAL neutrophils was significant as long as 51 days after initial dermal sensitization, with an apparent (not statistically significant) peak at 31 days.

Fig E2 (see this article's Online Repository at www.jacionline.org) shows the changes in MIP-2 and TNF- α concentrations in BAL 24 hours after intranasal instillation. Compared with the 0/0/0 control groups, the TDI-treated mice (1/1/1) showed an increased concentration of MIP-2 in BAL until 31 days after initial dermal sensitization. There were no differences in TNF- α levels between TDI-treated and vehicle-treated mice.

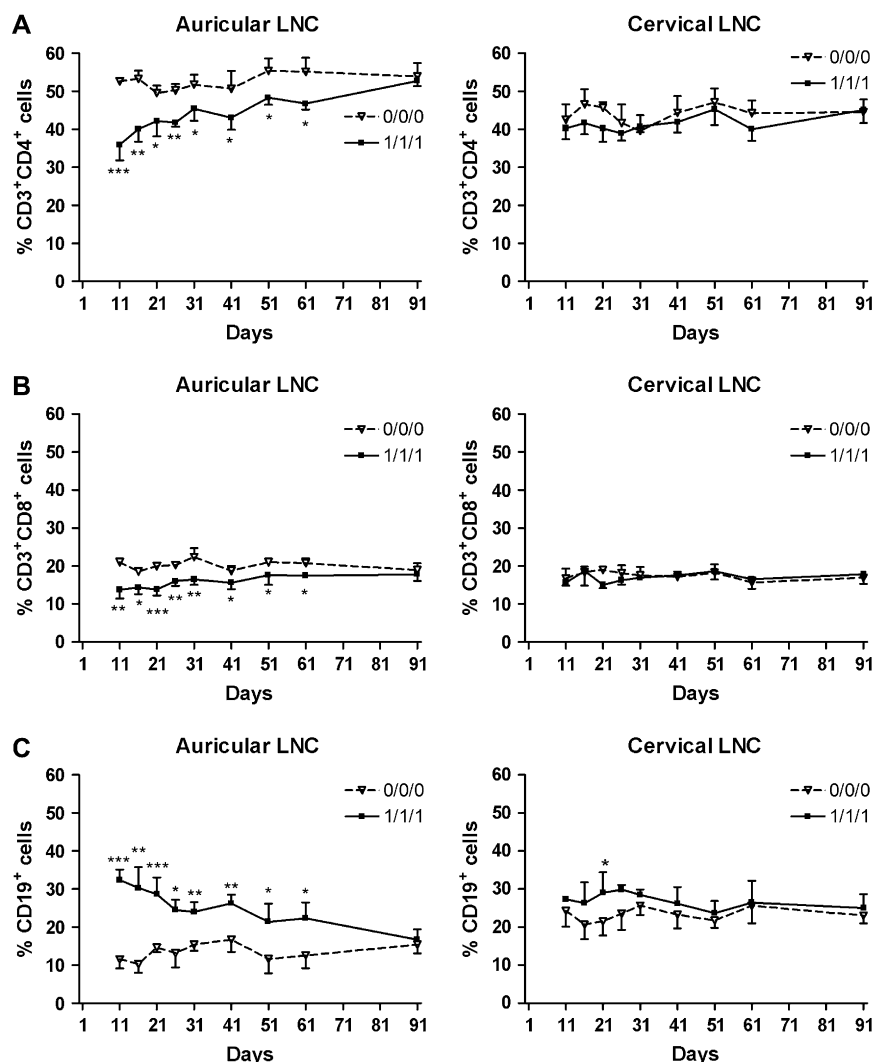


FIG 3. CD3⁺CD4⁺ (T_H), CD3⁺CD8⁺ (T-cytotoxic lymphocytes), and CD19⁺ (B lymphocytes) lymphocyte subpopulations in auricular (*left*) and cervical (*right*) lymph nodes 1 day after intranasal instillation of TDI or vehicle. All graphs show mean and SD. Lymph node cells (LNC) were stained with anti-CD3 (allophycocyanine), anti-CD4 (phycoerythrin; **A**) and anti-CD8 (peridinin-chlorophyll cyanine; **B**) or received a single staining with anti-CD19 (phycoerythrin; **C**)-labeled antibodies. Experimental groups are as in Fig 1. *n* = 3-4 experiments per group. **P* < .05; ***P* < .01; ****P* < .001 compared with the control group.

Lymphocyte subpopulation analysis

Fig 3 shows a decrease in the proportions of CD3⁺CD4⁺ cells (T-helper lymphocytes; *A, left*) and CD3⁺CD8⁺ cells (T-cytotoxic lymphocytes; *B, left*) and a increase in the proportion of CD19⁺ cells (B lymphocytes; *C, left*) in the auricular lymph nodes of TDI-treated mice as long as 61 days after initial dermal sensitization, compared with the vehicle-treated mice. Ninety days after the first contact with TDI, the proportional distribution of lymphocytes in the auricular lymph nodes of sensitized mice was the same as in the auricular lymph nodes of the vehicle-treated groups.

Almost no differences were found in the cervical lymph nodes of TDI-treated mice compared with vehicle-treated mice (Fig 3, *A-C, right*).

Lymphocytes of both auricular and cervical lymph nodes showed a spontaneous release (ie, without stimulation of ConA) of IL-4 and IFN- γ near the detection limit levels. There were no differences in basal release between the groups (Fig 4, data shown as a dotted line representing the mean of all measurements).

TDI-treated mice showed, as long as 21 days after initial contact, a significantly increased level of IL-4 in the supernatant of auricular lymph nodes after ConA stimulation compared with the control groups (Fig 4, *A, left*). The level of IFN- γ in ConA-stimulated, auricular lymphocytes of TDI-treated mice was significantly increased compared with the controls as long as 16 days after initial contact (Fig 4, *B, left*). ConA-stimulated cervical lymphocytes of TDI-treated mice only showed a significantly

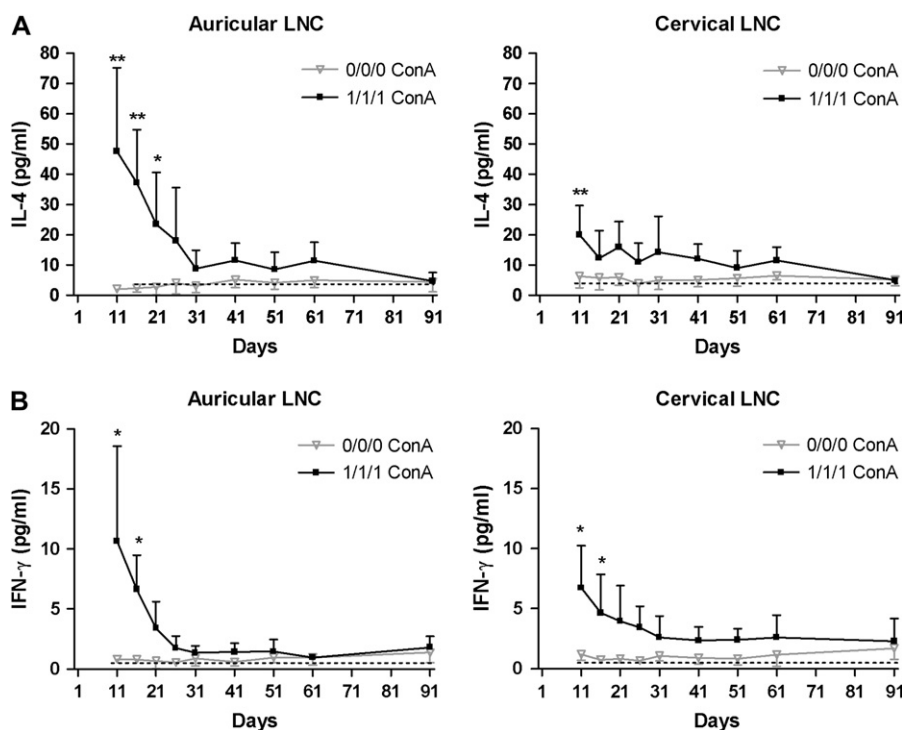


FIG 4. IL-4 (**A**) and IFN- γ (**B**) levels in auricular (*left*) and cervical (*right*) lymphocyte supernatant 1 day after intranasal instillation of TDI or vehicle. All graphs show mean and SD. Lymph node cells (LNC) were cultured (18 hours) without (dotted line representing the average spontaneous release) or with ConA (2.5 μ g/mL) stimulation. Experimental groups are as in Fig 1. $n = 3$ -4 experiments per group. * $P < .05$; ** $P < .01$ compared with the control group.

increased level of IL-4 as long as 11 days after first treatment (Fig 4, A, *right*) and a significantly increased level of IFN- γ as long as 16 days after first treatment compared with the controls (Fig 4, B, *right*).

Total serum immunoglobulins

TDI-treated mice showed significantly higher total serum IgE levels in all groups compared with the control mice. At day 11, a doubling of the total serum IgE levels was already found in TDI-treated mice. At later time points, total serum IgE levels increased further and then remained 2.5-fold to 3-fold higher than in the control group up to the last time point—that is, 91 days (Fig 5, A). Total serum IgG₁ (Fig 5, B) of TDI-treated mice started to increase later compared with IgE, becoming significant at 51 days after the first dermal sensitization, and continuing to increase until 91 days after initial dermal sensitization. Total serum IgG_{2a} (Fig 5, C) of TDI-treated mice also started later than IgE, becoming significant from 41 days after the first dermal sensitization, and remaining elevated until 91 days.

Fig E3 (see this article's Online Repository at www.jacionline.org) shows the total serum IgE, IgG₁, and IgG_{2a} 91 days after initial dermal sensitization. The data for the 0/0/0 and 1/1/1 group are identical to those shown in Fig 5, but results from additional 1/1/0 and 0/0/1 groups have been added. Total serum IgE, IgG₁, and IgG_{2a} levels

were significantly increased for both the 1/1/0 and 1/1/1 groups compared with the 0/0/1 and 0/0/0 control groups. There were no differences in immunoglobulin concentrations between the control groups (0/0/0 and 0/0/1), and between the 2 dermally sensitized groups (1/1/0 and 1/1/1).

Consistency among the various endpoints measured

Because several endpoints were assessed in each animal, it was possible to look for correlations at the individual mouse level as presented in Table I. Unfortunately, it was not possible to include the R measurements obtained with the forced oscillation technique, because these were performed in a separate set of mice. The lymph node data were also not included because lymph nodes from 2 to 3 mice were always pooled to obtain enough cells. Only the mice from the TDI-treated group were included in these calculations, because the inclusion of the control groups, which showed no changes, would have yielded significant but meaningless correlations.

The most important findings are (1) the physiologic airway responses (early response and airway hyperreactivity [AHR]) and the airway inflammation (total cell count, neutrophils and MIP-2 in BAL) depend on the day of challenge; (2) the magnitudes of the early response and AHR are positively correlated with the degree of airway

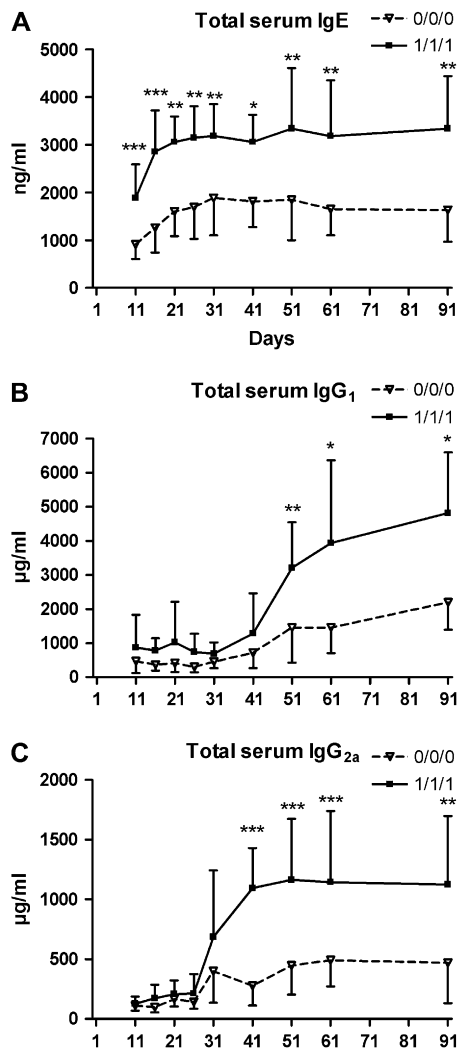


FIG 5. Total serum IgE (A), IgG₁ (B), and IgG_{2a} (C) levels 1 day after intranasal instillation of TDI or vehicle. All graphs show mean and SD of total immunoglobulin concentrations in serum. Note that the scale is different in the graphs: IgE = ng/mL; IgG₁ and IgG_{2a} = µg/mL. Experimental groups are as in Fig 1. n = 8–10. *P < .05; **P < .01; ***P < .001 compared with the control group.

inflammation (total cell count, neutrophils, and MIP-2) (3) but they are negatively correlated with the immunoglobulin concentrations in serum, indicating that an increase in IgE, IgG₁, and IgG_{2a} is not directly linked to adverse functional airway changes in this model.

DISCUSSION

Only few studies have investigated the time course of immunologic and functional changes after sensitization by reactive chemicals. As shown before,^{16–18} we found evidence of systemic sensitization (increased total serum IgE) already 11 days after initial dermal application of TDI. These observations are compatible with the notion that as a respiratory sensitizer, TDI is capable of inducing

a T_H2 cytokine profile in cells of the local draining lymph nodes.^{19,20} The increase in serum IgG₁, which occurs later, is also compatible with T_H2 stimulation in mice. However, the increase in serum IgG_{2a}, which showed the same trend as IgG₁, was unexpected because such increase is characteristic for T_H1 stimulation in mice. Such polyclonal immunoglobulin increase has been shown in other animal models of allergy, in which compounds of either high²¹ or low^{22,23} molecular weight were used. Thus Pochanke et al²¹ found increasing concentrations of total serum IgE, IgG₁, and IgG_{2a} starting day 15 after a fur mite infestation, with a plateau between day 50 and day 105.

The contribution of IgE in isocyanate-induced occupational asthma is still unclear, because specific IgE responses to isocyanates are detected in only a minority of isocyanate-induced asthma.²⁴ Isocyanates are very unstable haptens that require binding to carrier proteins forming a conjugate. It is debatable to what extent specific IgE antibody production is not detected because of inappropriate conjugates and to what extent the disease is mediated by alternative immunologic mechanisms.¹⁰ The role of IgG responses to occupational agents is even more complex. Serum-specific IgG may persist for many years after the last exposure to TDI,¹⁰ as also shown in our results obtained in mice, although it needs to be considered that we measured total serum IgG and not specific IgG. Furthermore, it has also been suggested that IgG₁ could be used to monitor the effect of exposure to diisocyanates before disease appears,²⁵ which we can not confirm in our model. Moreover, although we do not have the evidence, it could be conceivable that IgG, which appears when airway responses and inflammation disappear, yield a protective effect in this model of OA.^{26,27}

To assess the effect of an intranasal instillation of TDI *per se* on these humoral parameters, we added 2 extra groups at the last time point (90 days) of our study: a non-sensitized group receiving intranasal TDI (0/0/1) and a TDI-sensitized group receiving vehicle only (1/1/0). The humoral responses were absent in both the 0/0/0 and the 0/0/1 groups, and the increases were similar in the 1/1/0 and 1/1/1 groups (see this article's Fig E3 in the Online Repository at www.jacionline.org), indicating that they were caused by the initial dermal sensitization to TDI, without any substantial effect of the intranasal instillation.

Although we found indications of systemic sensitization until 90 days after initial dermal sensitization, the auricular lymph nodes progressively returned to a control cellular distribution and *in vitro* IL-4 and IFN-γ production, with increasing time between dermal treatment and intranasal challenge. These changes in the local draining lymph nodes parallel the respiratory and inflammatory responses better (though not perfectly) than those in the serum immunoglobulins. Previously, we have demonstrated that the lymphocytes are important in chemical-induced asthma, given that we were not able to elicit respiratory responses and inflammatory findings in severe combined immunodeficiency mice.¹⁸ Furthermore, the importance of both CD4⁺ (T_H) and CD8⁺ (T-cytotoxic lymphocyte)

TABLE I. Nonparametric Spearman correlation for data from the TDI-sensitized and challenged group (1/1/1)†

Day of challenge	Airway response		BAL					Total serum immunoglobulin		
	Early response	AHR	Total cell count	Macrophages	Neutrophils	MIP-2	TNF- α	IgE	IgG ₁	IgG _{2a}
Day of challenge	−0.47***	−0.54***	−0.30**	−0.10	−0.35***	−0.25*	−0.15	0.39***	0.63***	0.81***
Airway response										
Early response		0.72***	0.31**	0.01	0.44***	0.29**	0.07	−0.20	−0.43***	−0.40***
AHR			0.36*	0.06	0.45**	0.17	0.01	−0.06	−0.21	−0.41**
BAL										
Total cell count				0.62***	0.63***	0.21	0.23*	−0.18	−0.29**	−0.27*
Macrophage					−0.12	−0.17	0.09	−0.09	0.03	−0.06
Neutrophil						0.35***	0.20	−0.19	−0.42***	−0.36***
MIP-2							0.02	−0.10	−0.22*	−0.11
TNF- α								−0.26*	−0.17	−0.05
Total serum immunoglobulin										
IgE									0.28**	0.20
IgG ₁										0.53***
IgG _{2a}										

* $P < .05$; ** $P < .01$; *** $P < .001$ (2-tailed).†Figures are r values; $n = 86$ for all parameters, except for AHR $n = 44$. Early response = AUC of Penh against time; AHR = AUC of Penh against methacholine concentration; macrophage = total number of macrophages in BAL; neutrophil = total number of neutrophils in BAL; MIP-2 = MIP-2 in BAL; TNF- α = TNF- α in BAL; IgE = total serum IgE; IgG₁ = total serum IgG₁; IgG_{2a} = total serum IgG_{2a}.

lymphocytes in mouse models of isocyanate asthma is generally accepted.^{15,28} The novel observations of our study are that the relative changes in CD4⁺, CD8⁺, and CD19⁺ lymphocytes in the auricular lymph nodes are limited in time, and that normalization in these parameters is accompanied by a decreasing asthmatic response after intranasal challenge. Admittedly, in this model with 1 intranasal instillation, the influence of the cervical lymph nodes appears rather limited.

The early ventilatory response and methacholine responsiveness seemed to be highest at 15 days (Fig 1), but this apparent peak was not statistically significant, mainly because of the large range in the magnitude of responses at all time points. Such large variation was also observed in our previous experiments^{18,29} and by other research groups³⁰ and was not simply a result of noisy measurements, but of true individual variation in biological responses, because the magnitudes of the changes in the various endpoints were consistent within each animal (Table I). The decreasing trend with time in ventilatory response and methacholine responsiveness was, however, significant.

The ventilatory responses were accompanied by an influx of neutrophils (and the chemokine MIP-2), which appeared to be maximal at the same time point and showed a similar decrease over time. Such neutrophil inflammation is not contradictory with allergic asthma, because neutrophilic inflammation has also been observed in patients with asthma triggered by TDI³¹ and other models of asthma. However, the nature (and kinetics) of the pulmonary inflammation in asthma is presumably dependent on the time course of the disease and the pattern of the exposure. This is an issue that we are currently investigating further.

What are the possible clinical correlates or implications of these observations made in a mouse model of

immunologically mediated chemical-induced asthma? One implication following from these animal data is that the respiratory responses to TDI, and hence TDI-induced OA, are mechanistically only loosely associated, if at all, with changes in circulating immunoglobulins, because the immunoglobulins were most manifest at a time when the respiratory and pulmonary inflammatory changes had returned to normal. This concept is not novel.²⁴ A further, more novel implication relates to the persistence of sensitization to the causal agent and symptoms of asthma in subjects with OA even after their removal from exposure. Here, our experiments match the human situation only partially. The progressive reduction in responsiveness with time is similar to what happens in patients with OA who cease to be exposed, because it is well established that subjects with OA generally become less responsive to the sensitizer after complete removal from exposure^{1-4,7,32}; however, it is not frequent for such patients to become completely unresponsive to the sensitizer, and a considerable fraction remain symptomatic with airway inflammation (with eosinophils and neutrophils).⁹ This was proven in an epidemiologic study in which the authors were able to prove that failure to improve after cessation of exposure to an agent causing OA, which was the case in 28 of the 98 subjects included in the study, is associated with airway inflammation—more specifically, increased numbers of sputum neutrophils, levels of IL-8, and neutrophil-derived myeloperoxidase at the time of follow-up. Thus, persisting inflammation is the cause of prolonged asthmatic symptoms.⁹ In another study, a population of spray painters with OA caused by TDI was studied. Months and even years after exposure, 7 of the 25 patients still showed immediate response to TDI exposure, and 8 no longer reacted but still showed clear symptoms of asthma.³² The authors of this study concluded that airway

sensitization to TDI and symptoms and functional airway abnormalities of asthma can persist for years after cessation of exposure and may have different outcome. In our mouse model, it remains to be established whether our mice, which were challenged only once, would become responsive again (and show lung inflammation) if repeated challenges were given at the later time points.

In our experiments, we showed that both specific and nonspecific airway responses decrease with increasing time between sensitization and challenge. Nevertheless, the mice show clear signs of systemic sensitization when the mice are re-exposed to very low concentrations. This implies that dermal contact with a chemical can lead to a lifelong sensitization and a possible reoccurrence of asthmatic symptoms.

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METHODS

Reagents

Toluene-2,4-diisocyanate (98%; Fluka, CAS 584-84-9) and acetyl-B-methylcholine (methacholine) were obtained from Sigma-Aldrich (Bornem, Belgium). Acetone and diethyl ether were obtained from VWR (Leuven, 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 volumes of acetone and 3 volumes of olive oil (Selection de Almazara, Carbonell, Madrid, Spain). Concentration of TDI is given as percent (vol/vol) in AOO.

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-hour 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.

Groups of animals and treatment protocol

The mice received dermal applications of 20 μ L vehicle or 20 μ L 0.3% TDI on each ear on days 1 and 8. On day 10, 15, 20, 25, 30, 40, 50, 60, or 90, they received under light diethyl ether anesthesia a single intranasal instillation of 10 μ L vehicle or 10 μ L 0.1% TDI in each nostril. In all experiments, treatment with TDI is indicated with 1, and treatment with the vehicle is indicated as 0. All experimental groups are indicated with 3 symbols: the first and second symbol indicate the dermal treatment on days 1 and 8, whereas the third symbol indicates the intranasal instillation. Thus, the 1/1/1 group consists of mice that received dermal applications of TDI on days 1 and 8 and intranasal instillations of TDI, whereas the 0/0/0 control group consists of mice that received AOO on all occasions. For the last time point (day 90) we included 2 extra groups: a group receiving dermal treatments with the vehicle and an intranasal instillation with TDI (0/0/1) and a group receiving dermal sensitization with TDI and an intranasal instillation with the vehicle (1/1/0).

Ventilatory function

Early ventilatory response and AHR. On the day of intranasal challenge, the ventilatory function of each mouse was first recorded in resting condition and unrestraint for 5 minutes in a whole-body plethysmograph (EMKA Technologies, Paris, France). Immediately after the intranasal instillation, mice were placed again in the whole-body plethysmograph (0 time point), and their ventilatory parameters were measured for 40 minutes. Every 30 seconds, the mean enhanced pause (Penh), which is a composite index indicative of airway obstruction, was calculated. The AUC of Penh against time between 0 and 40 minutes was calculated for each individual mouse, and this figure was used for statistical analysis.

One day after intranasal challenge—that is, 22 hours—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 minute, of nebulized methacholine (successively 0, 5, 10, 25, and 50 mg/mL). An average Penh over a period of 30 seconds was calculated during 3 minutes (6 measurements), and the mean of these 6 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 performed to validate the findings obtained by whole-body plethysmography, airway responses were measured by using a forced oscillation technique (FlexiVent;

SCIREQ, Montreal, Canada). This was done in a separate group of mice, and only selected time points were performed: 15, 30, 60, and 90 days after initial dermal sensitization. One day after intranasal challenge—that is, 22 hours—measurement of airway resistance (R) to methacholine was assessed. Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg/kg). The trachea was exposed and tracheotomized, and an 18-gauge metal needle was inserted. Mice were connected to a computer-controlled small animal ventilator. The mice were quasisinusoidally ventilated with a tidal volume of 10 mL/kg at a frequency of 150 breaths/minute 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 seconds, with increasing concentrations (0, 0.625, 1.25, 2.5, 5, and 10 mg/mL). R was measured using a snapshot protocol every 20 seconds for 2 minutes. The mean of these 6 values was used for each methacholine concentration unless the coefficient of determination of a measurement was smaller than 0.95. These data were excluded by the FlexiVent software v5.1 (Scireq, Montreal, Quebec, Canada). For each mouse, R was plotted against methacholine concentration (from 0 to 10 mg/mL), and the AUC was calculated.

Pulmonary inflammation (BAL)

One day after intranasal instillation (24 hours), mice were deeply anesthetized by intraperitoneal injection of pentobarbital (90 mg/kg body weight) and killed. Blood was first sampled from the retro-orbital plexus, and the mice were then killed by section of the abdominal vessels. The lungs were lavaged, *in situ*, 3 times with 0.7 mL sterile saline (0.9% NaCl), and the recovered fluid was pooled. Cells were counted by using a Bürker hemocytometer (total cells), and the BAL fluid was centrifuged (1000g, 10 minutes). The supernatant was frozen (–80°C) until further analyses. For differential cell counts, 250 μ L of the resuspended cells (100,000 cells/mL) were spun (1400g, 6 minutes; 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 TNF- α and MIP-2 were measured in undiluted BAL fluid by a sandwich ELISA according to the manufacturer's instructions (TNF- α : Biosource, Nivelles, Belgium; MIP-2: R&D Systems, Abingdon, United Kingdom). Lower limits of detection were 3.0 pg/mL and 1.5 pg/mL for TNF- α and MIP-2, respectively.

Lymph node cells

Retroauricular and superficial cervical lymph nodes were also obtained from the same mice 24 hours after intranasal instillation. 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 μ m; BD Biosciences, Erembodegem, Belgium) and rinsing with 10 mL tissue culture medium (RPMI-1640). Cells were counted by using a Bürker hemocytometer. Lymphocytes were washed 3 times and suspended (10^7 cells/mL) in complete tissue culture medium (RPMI-1640 supplemented with 10% heat-inactivated FBS, 10 mg/mL streptomycin, 100 IU/mL penicillin, 1 mmol/L sodium pyruvate, nonessential amino acids).

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

control samples being labeled with isotype match control antibodies (BD Biosciences). Flow cytometry (Facsarray; BD Biosciences) was performed by using at least 10^4 cells.

Cells were also seeded into 48-well culture plates at a density of 2×10^6 cells/mL and incubated in complete RPMI-1640 medium for 18 hours without or with 2.5 μ g/mL ConA (Sigma-Aldrich, Bornem, Belgium). Cells were then centrifuged (2000g, 10 minutes), and supernatants were stored at -80°C . Concentrations of IL-4 and IFN- γ were measured in undiluted supernatant by standard ELISA technique, 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.

Mouse total serum IgE, IgG₁, and IgG_{2a}

The OptEIA Mouse IgE and IgG_{2a} set from Pharmingen (BD Biosciences) 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), we coated our plates using purified antimouse IgG₁, a standard was created by using purified mouse IgG₁, and the further measurements were performed according to the manufacturer's (BD Pharmingen) instructions with the use of biotinylated antimouse IgG₁ and avidin horseradish peroxidase conjugate.

Data analysis

Data are shown as means and SDs. All data were analyzed by using the nonparametric Mann-Whitney *t* test with a 95% CI. Results of total serum IgE, IgG₁, and IgG_{2a} at day 91 were analysed by using the nonparametric ANOVA test, Kruskal-Wallis, with a 95% CI (GraphPad Prism 4.01, GraphPad Software Inc, San Diego, Calif). A level of $P < .05$ (2-tailed) was considered significant. Nonparametric Spearman correlation test fitting was performed with SAS8.2 software, (SAS Institute Inc, Tervuren, Belgium) to describe correlations among day of challenge, early response, response to methacholine

challenge, total BAL cell count, total number of macrophages in BAL, total number of neutrophils in BAL, MIP-2 and TNF- α levels in BAL, and total serum IgE, IgG₁, and IgG_{2a} levels in the TDI-sensitized and challenged group.

RESULTS

Body weight

Fig E1 shows the weight of the mice before autopsy—that is, 24 hours after intranasal challenge. Mice that received TDI showed a significantly decreased body weight as long as 60 days after initial dermal treatment, compared with the mice that were treated with the vehicle.

DISCUSSION

Unexpectedly, we found clear differences in the weight of the mice just before autopsy (Fig E1). Presumably, we are not the only researchers weighing the mice before autopsy. Nevertheless, these observations are not often included in the results. Because all mice had the same age and weight (6 weeks and ± 20 g) at the beginning of the experiment and because all experimental groups were constituted randomly, the mean weight of all groups were presumably similar, so this effect on body weight probably represents a true effect of sensitization and challenge. Admittedly, we do not have the weight of the mice before challenge, so we can not rule out a possible irritant effect of the TDI intranasal challenge and of the subsequent early ventilatory response on the food intake of the mice during the following 24 hours.

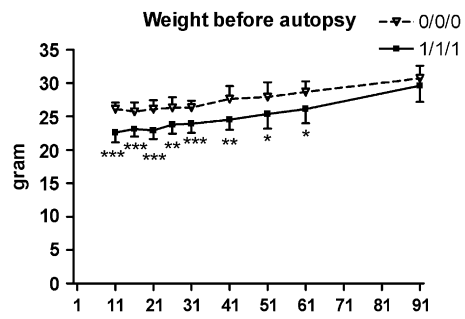


FIG E1. Weight of the mice 24 hours after intranasal instillation (before autopsy) of TDI or vehicle. Body weight is presented as mean and SD. Experimental group legends are as in Fig 1. n = 8-10. * $P < .05$; ** $P < .01$; *** $P < .001$ compared with the control group.

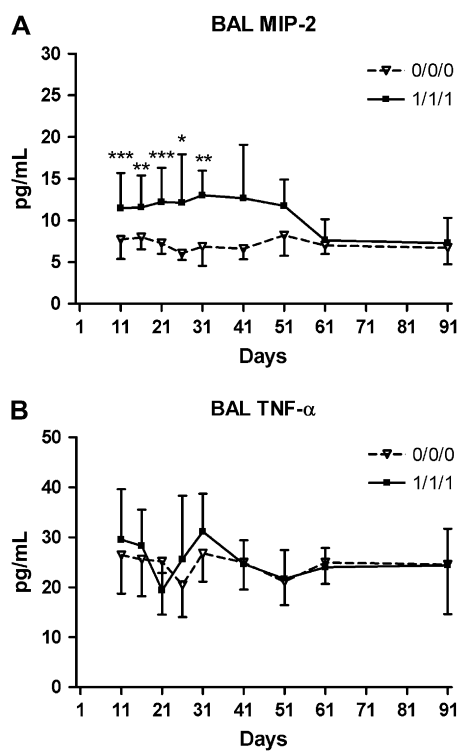


FIG E2. MIP-2 (**A**) and TNF- α (**B**) in BAL 24 hours after intranasal instillation of TDI or vehicle. Graphs show mean and SD. Experimental group legends are as in Fig 1. $n = 8-10$. * $P < .05$; ** $P < .01$; *** $P < .001$ compared with the control group.

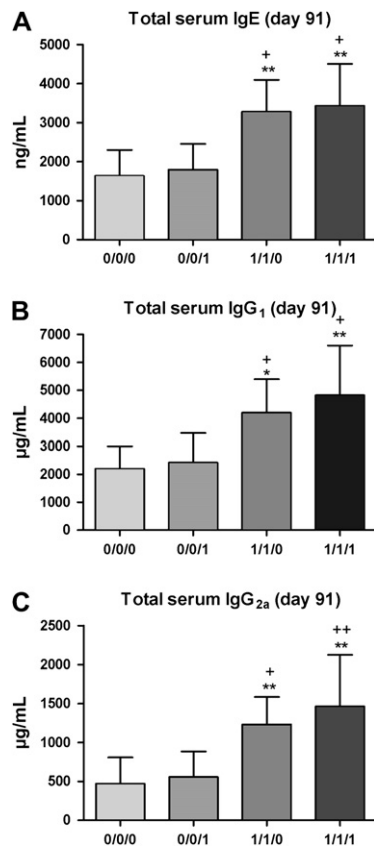


FIG E3. Total serum IgE (A), IgG₁ (B), and IgG_{2a} (C) levels 1 day after intranasal instillation of TDI or vehicle on day 90. All graphs show mean and SD of total serum immunoglobulin concentration in serum collected 24 hours after the intranasal challenge on day 90. Experimental groups are as in Fig 1, with inclusion of 2 extra groups: 0/0/1 (dermally treated with vehicle, intranasally challenged with TDI) and 1/1/0 (dermally treated with TDI, intranasally challenged with vehicle). $n = 8-10$. + $P < .05$, ++ $P < .01$ compared with the 0/0/1 control group. * $P < .05$, ** $P < .01$ compared with the 0/0/0 control group.