



Neuro-immune interactions in chemical-induced airway hyperreactivity

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ABSTRACT Asthma may be induced by chemical sensitizers, *via* mechanisms that are still poorly understood. This type of asthma is characterised by airway hyperreactivity (AHR) and little airway inflammation. Since potent chemical sensitizers, such as toluene-2,4-diisocyanate (TDI), are also sensory irritants, it is suggested that chemical-induced asthma relies on neuro-immune mechanisms.

We investigated the involvement of transient receptor potential channels (TRP) A1 and V1, major chemosensors in the airways, and mast cells, known for their ability to communicate with sensory nerves, in chemical-induced AHR.

In vitro intracellular calcium imaging and patch-clamp recordings in TRPA1- and TRPV1-expressing Chinese hamster ovarian cells showed that TDI activates murine TRPA1, but not TRPV1. Using an *in vivo* model, in which an airway challenge with TDI induces AHR in TDI-sensitized C57Bl/6 mice, we demonstrated that AHR does not develop, despite successful sensitization, in *Trpa1* and *Trpv1* knockout mice, and wild-type mice pretreated with a TRPA1 blocker or a substance P receptor antagonist. TDI-induced AHR was also abolished in mast cell deficient *Kit^{W^{sh}/W^{sh}}* mice, and in wild-type mice pretreated with the mast cell stabiliser ketotifen, without changes in immunological parameters.

These data demonstrate that TRPA1, TRPV1 and mast cells play an indispensable role in the development of TDI-elicited AHR.



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Chemical-induced AHR relies on neuro-immune interactions, involving lymphocytes, TRP channels and mast cells <http://ow.ly/Z4Lth>

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Introduction

Asthma is a chronic airway disease [1] which can be distinguished into various (overlapping) categories, depending on aetiology or patterns of airway inflammation [2, 3].

Most experimental asthma research has focused on atopic asthma, such as that caused in adults or children by IgE-mediated allergy to biological agents, most often using models in which mice or other animals are sensitised to ovalbumin (OVA) or other proteins [4, 5]. However, up to half of people with asthma are not atopic [6] and the aetiology and pathophysiology of this type of asthma remain largely unelucidated. In this regard, one possible avenue of research is that of asthma caused by exposure to reactive chemicals [7], as may occur in the workplace. Such chemical-induced occupational asthma has similar clinical features to atopic asthma, but the immune and nonimmune processes leading to this form of asthma have been less well investigated [8].

In addition, asthma can present with varying types and degrees of airway inflammation, as evidenced by the cellular composition of sputum [2]. Atopic asthma is traditionally characterised by eosinophil inflammation, but other patterns exist as well. One of these subtypes is paucigranulocytic asthma, which differs from classic atopic asthma by a low degree of airway inflammation. According to SIMPSON *et al.* [9], in 30% of asthma cases airway hyperreactivity (AHR) can exist in the absence of inflammatory cells in the airways. The mechanisms of this form of asthma are not well understood.

We undertook the present mechanistic study to improve our understanding of the pathophysiology of a clinically important fraction of asthma, namely asthma that is not dependent on IgE responses and not characterised by much cellular inflammation in the airways. For this we have used a well-validated mouse model of chemical-induced immune-mediated asthma based on sensitisation to toluene-2,4-diisocyanate (TDI), a well-described occupational asthmogen [8, 10–12].

Diisocyanates, including TDI, have a long history of use for a large number of industrial applications, and they represent a leading cause of chemical-induced occupational asthma [13, 14]. A recent longitudinal study of workers exposed to low airborne levels of TDI at a modern polyurethane foam factory in Eastern Europe has shown that over the first year of follow-up 14% of workers developed respiratory symptoms, TDI-specific IgGs and/or changes in spirometry, all indicative of early TDI-related health effects. Moreover, workers that were lost to follow-up (25%) were more likely to have had asthma symptoms [15]. Another recent study, evaluating the long-term outcome of diisocyanate-induced asthma, has shown that 50% of diisocyanate asthmatics remain symptomatic, with worsening of lung function and need of continuous anti-asthmatic therapy after an exposure-free period of 12 years, thus indicating the long-term morbidity of occupational asthma caused by diisocyanates [16].

Several researchers have sought to discover individual risk factors and mechanisms leading to TDI-induced asthma in humans. An important observation has been that the risk of TDI-induced asthma was associated with a polymorphism of the neurokinin 2 receptor (NK2R), the receptor for neuropeptides that are released from activated airway sensory neurons, thus suggesting a neurogenic mechanism in TDI-induced asthma. This NK2R 7853G>A polymorphism was associated with higher serum vascular endothelial growth factor levels, and thus airway inflammation in TDI-exposed workers [17]. In healthy human subjects, activation of transient receptor potential channels (TRP)A1 or TRPV1, *via* inhalation of irritant agonists (cinnamaldehyde or capsaicin), has been shown to evoke cough, a reflexive airway response, which may also contribute to asthmatic symptoms [18]. Capsaicin treatment has been shown to reduce nasal hyperreactivity in patients with idiopathic rhinitis, *via* ablation of the TRPV1-substance P nociceptive signalling pathway in the nasal mucosa, providing further evidence for an involvement of TRP and neurogenic inflammation in human respiratory diseases [19].

Besides being a potent sensitiser, TDI is an extremely potent sensory irritant. Exposure to TDI leads to stimulation of trigeminal nerve endings present in respiratory and nasal mucosa [20, 21], thereby inducing the local release of tachykinin neuropeptides, including substance P, neurokinin A and calcitonin-gene-related peptide [22, 23]. This probably results from stimulation of TRP channels [24].

Since TDI is both a potent immune sensitiser and sensory irritant, this chemical agent provides a unique opportunity to investigate the interaction between immunological and neurological processes. It has indeed been proposed that asthma is an immune-neuronal disorder [25], like other inflammatory conditions [26]. However, the link between activation of neuronal receptors and immune responses is not well established in asthma.

Combining our mouse model of chemical-induced asthma with *in vivo* and *in vitro* studies of TRPA1 and TRPV1 activation [27–29], we tested the hypothesis that stimulation of sensory TRP channels critically modulates chemical-induced airway responses. We also investigated the role of mast cells, because mast cells are located in the vicinity of sensory nerve fibres in the airways [25] and they can thus immediately respond to released neuropeptides.

Here, we first demonstrate that TDI activates TRPA1, but not TRPV1 *in vitro*, and then that in our *in vivo* model, activation of TRPA1 (direct) and TRPV1 (indirect), together with activation of mast cells, contributes to the development of AHR. We conclude that neuro-immune interactions, involving TRPA1, TRPV1 and mast cells, are crucial in developing nonatopic irritant-induced AHR, even in the absence of cellular manifestations of inflammation.

Materials and methods

A complete description of the materials and methods is available in the online supplementary material.

In vitro experiments

TDI-induced activation of TRPA1 or TRPV1 was investigated by intracellular calcium (Ca^{2+}) imaging and patch-clamp experiments on Chinese hamster ovarian (CHO) cells selectively expressing murine (m) TRPA1 or mTRPV1. A tetracycline-regulated system was used for inducible expression of TRPA1 or TRPV1 in CHO cells. TRPA1 currents were recorded in the cell-attached patch-clamp configuration.

Experimental protocols of mouse experiments

All experimental procedures performed in mice were approved by the KU Leuven local ethics committee for animal experiments (P166-2012).

Our model of chemical-induced asthma, as described previously in Balb/c mice [11], was applied to C57Bl/6 wild-type mice. Briefly, mice were sensitised to TDI by applying 20 μL TDI (1% in acetone/olive oil (AOO)) to the dorsum of both ears on days 1 and 8; nonsensitised controls received similar applications of the vehicle only. On day 15, mice were challenged by means of an intranasal instillation of 30 μL of 0.1% TDI or vehicle (AOO). 1 day later, the main physiological outcome, *i.e.* nonspecific airway reactivity to methacholine, was measured using the flexiVent (Scireq, Montreal, QC, Canada) [30], and the following immunological readouts were obtained: cell counts and distribution in bronchoalveolar lavage (BAL) fluid, total serum IgE, lymphocyte subpopulations in retro-auricular lymph nodes and their *ex vivo* cytokine release.

We compared the responses obtained in wild-type mice to those obtained in knockout mice devoid of TRPA1 (*Trpa1*^{-/-}), TRPV1 (*Trpv1*^{-/-}), mast cells (*Ki1^{Wsh/Wsh}*) or lymphocytes (*Rag2*^{-/-}). We also tested responses in wild-type mice having received pharmacological agents before the challenge with TDI: HC030031 (TRPA1 antagonist), RP67580 (substance P receptor antagonist) or ketotifen (mast cell stabiliser); or during the challenge: allyl isothiocyanate (TRPA1 agonist) or capsaicin (TRPV1 agonist).

Statistical analysis

Electrophysiological measurement data were analysed using the WinASCD software package (KU Leuven, Leuven, Belgium). Dose–response curves (AHR) were analysed using two-way parametric ANOVA, followed by a Bonferroni multiple comparison *post hoc* test. All other data were analysed using one-way parametric ANOVA, followed by a Bonferroni multiple comparison *post hoc* test.

Results

TDI activates TRPA1 *in vitro*

In vitro Ca^{2+} imaging experiments showed that 0.001% TDI caused a significant increase in intracellular Ca^{2+} concentration in mTRPA1-expressing CHO cells, but not in mTRPV1-expressing cells (online supplementary fig. S1). 0.001% toluene diamine (TDA), which is the hydrolysis product of TDI, containing two amine groups instead of reactive isocyanate groups, was ineffective on both mTRPA1- and mTRPV1-expressing cells (online supplementary fig. S1).

Furthermore, cell-attached patch-clamp recordings in mTRPA1-expressing CHO cells showed that the application of 0.001% TDI leads to enhanced channel activation (fig. 1a–c) and an increase in the mean current (fig. 1e). The presence of functional TRPA1 was confirmed by applying 100 μM menthol together with TDI, which causes a “flickering” of the current (fig. 1a, d) that is characteristic of the blocking effect of menthol on TRPA1 [27]. Taken together, these data demonstrate that TDI directly activates TRPA1, but not TRPV1.

TDI causes AHR only after prior sensitisation to TDI

Intranasal instillation of 0.1% TDI induced AHR to methacholine 24 h later, only if mice had been previously sensitised to TDI (TDI/TDI) (fig. 2a; for individual responses see online supplementary fig. S2). TDI-sensitised and TDI-challenged mice showed increased levels of total serum IgE (fig. 2b), increased numbers of CD4⁺ T-helper (Th)-cells, CD8⁺ cytotoxic T-cells and B-cells (CD19⁺) in the auricular lymph nodes draining the site of sensitisation (fig. 2c), along with increased releases of both Th2 (interleukin

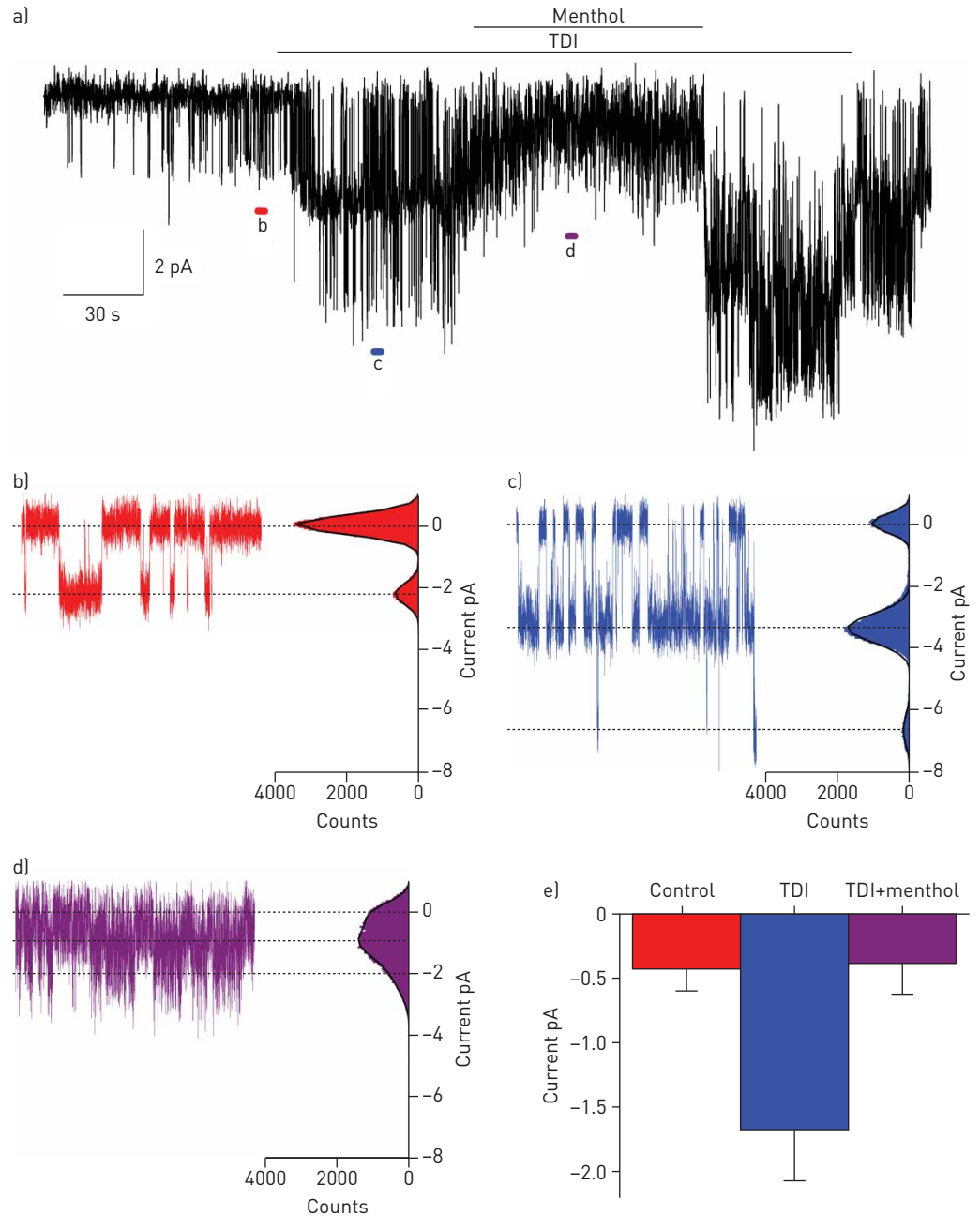


FIGURE 1 Toluene-2,4-diisocyanate (TDI) activates transient receptor potential A1 channels. a) Representative cell-attached patch-clamp recording showing the effect of extracellular application of 0.001% TDI and 100 μ M menthol; b–d) magnification of corresponding traces and all-point amplitude histograms for each condition; e) mean \pm SEM current during 30 s of each condition.

(IL)-13 and IL-10) and Th1 (interferon (IFN)- γ) cytokines upon incubation of these lymphocytes with concanavalin A *in vitro* (fig. 2d). Thus, these mice showed immunological changes, confirming that they had been immunologically sensitised [31].

Of note, the AHR in TDI-sensitised and TDI-challenged mice was not accompanied by increases in inflammatory cells (neutrophils, eosinophils or lymphocytes) in BAL fluid (fig. 2e). In other words, TDI induced sensitisation-dependent AHR without signs of cellular inflammation in the airways in C57Bl/6 mice.

Involvement of TRPA1 and TRPV1 in TDI-induced AHR

To test whether TRPA1 or TRPV1 has a role in generating AHR, we compared the methacholine responses of *Trpa1* and *Trpv1* knockout mice with those of similarly and concurrently treated wild-type

mice. In contrast to wild-type mice, both TDI-sensitised and TDI-challenged *Trpa1* knockout and *Trpv1* knockout mice did not develop AHR (fig. 3a and fig. 4a; for individual responses see online supplementary fig. S2). This lack of an AHR response in both *Trpa1* and *Trpv1* knockout mice was not due to absence of sensitisation, because their immune responses did not differ from those of similarly treated wild-type mice (fig. 3b–d and fig. 4b–d).

To confirm the role of TRPA1, we assessed the effect of pharmacological inhibition of the TRPA1 receptor. As seen in *Trpa1* knockout mice, TDI-sensitised wild-type mice pretreated with a selective TRPA1 blocker (HC030031) 30 min prior to the TDI challenge, did not develop AHR (fig. 5a; for individual responses see online supplementary fig. S2), although immune-sensitisation was not affected (online supplementary fig. S3a and b).

Since activation of TRPA1 and TRPV1 is known to induce the release of the neuropeptide substance P [32], we sought to determine whether substance P is involved in the observed TDI-induced respiratory responses. TDI-sensitised wild-type mice, having received an intraperitoneal injection of RP67580, an antagonist of the neurokinin 1 receptor (NK1R; the receptor for substance P), 30 min prior to the TDI challenge, did not develop AHR 24 h later (fig. 5b; for individual responses see online supplementary fig. S2). Again, this could not be attributed to the NK1R antagonist having abolished immune-sensitisation (online supplementary fig. S3c–e).

Thus, contrary to control wild-type mice, mice deficient in TRPA1 or TRPV1 or wild-type mice in which the TRPA1 channel or the receptor responding to neuropeptides released by sensory neurons had been blocked, did not develop AHR in response to a TDI challenge, even though they had been successfully sensitised to TDI.

These results provide evidence for a crucial role of TRPA1 and TRPV1 activation in TDI-induced AHR. Nevertheless, to exclude that the mere activation of these TRP channels leads to AHR in TDI-sensitised

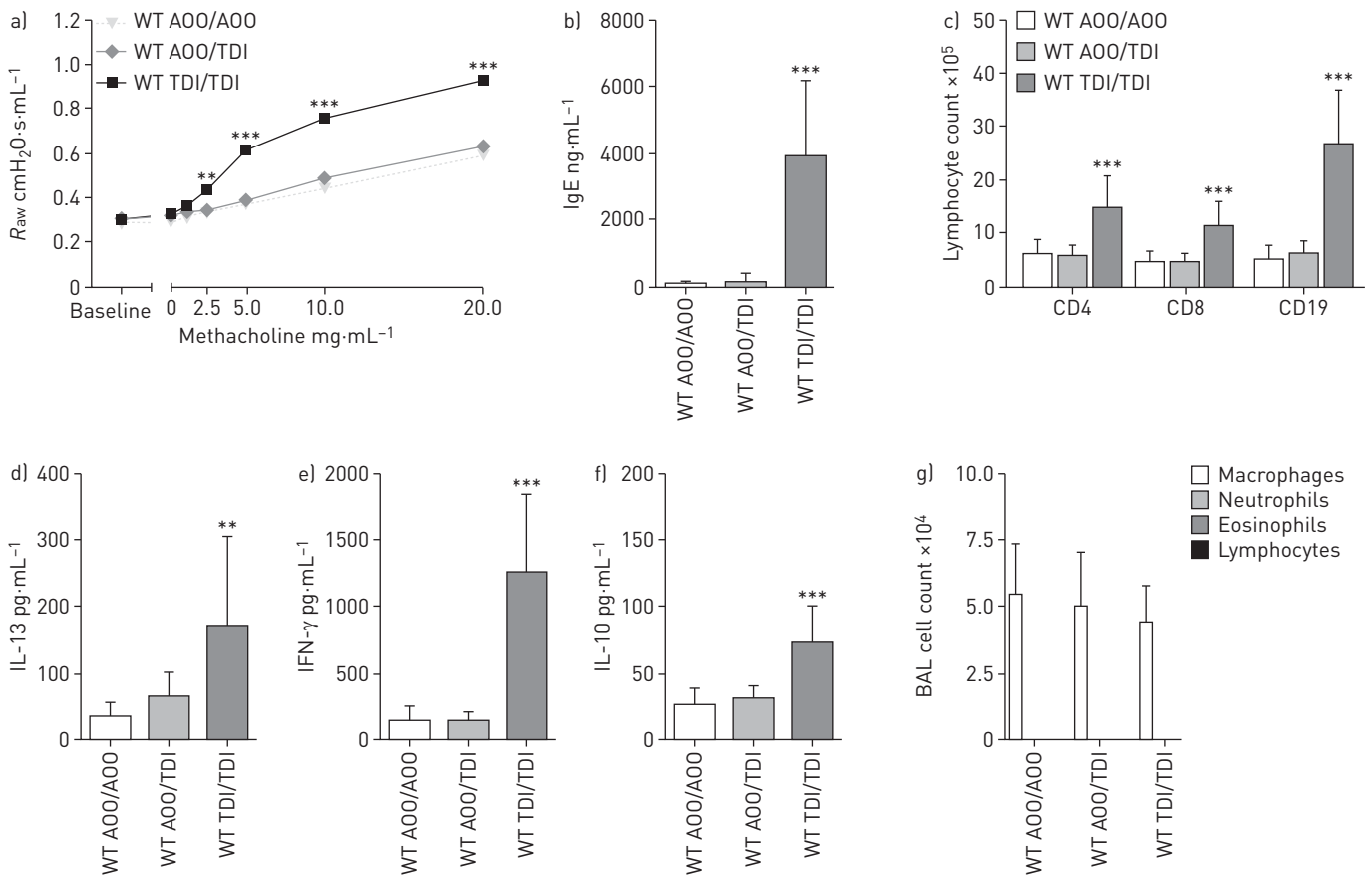


FIGURE 2 Toluene-2,4-diisocyanate (TDI) causes airway hyperresponsiveness after prior sensitisation. a) Airway resistance (R_{aw}) in response to methacholine; b) total serum IgE levels; c) number of T-helper cells (CD4⁺), cytotoxic T-cells (CD8⁺) and B-lymphocytes (CD19⁺); cytokine levels of d) interleukin (IL)-13, e) interferon (IFN)- γ and f) IL-10; g) differential cell count in the bronchoalveolar lavage (BAL) fluid. Data are presented as mean \pm sd, n=6–9 per group. AOO: acetone/olive oil; WT: wild-type. **: p<0.01; ***: p<0.001 compared to WT AOO/AOO control group.

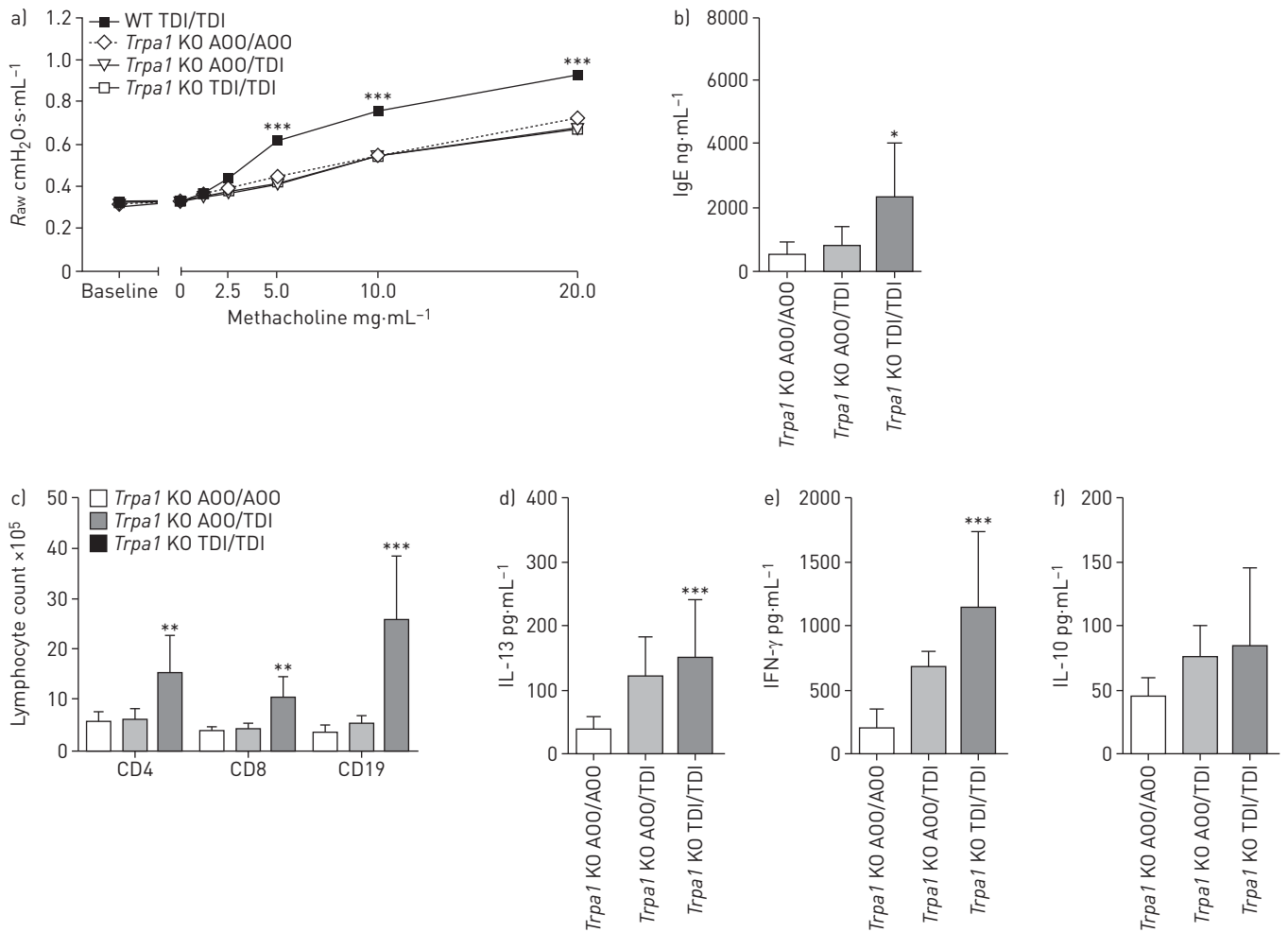


FIGURE 3 Involvement of transient receptor potential A1 (TRPA1) channels in toluene-2,4-diisocyanate (TDI)-induced airway hyperresponsiveness: *Trpa1* knockout (KO) mice. a) Airway resistance (R_{aw}) in response to methacholine in *Trpa1* KO mice; b) total serum IgE levels; c) number of T-helper cells (CD4⁺), cytotoxic T-cells (CD8⁺) and B-lymphocytes (CD19⁺); cytokine release of d) interleukin (IL)-13, e) interferon (IFN)- γ and f) IL-10. Data are presented as mean \pm SD, n=5–8 per group. WT: wild-type; AOO: acetone/olive oil. *: p<0.05; **: p<0.01; ***: p<0.001 compared to *Trpa1* KO AOO/AOO control group.

subjects, we substituted the intranasal challenge with TDI with an intranasal instillation of allyl isothiocyanate (AITC), a potent agonist of TRPA1 and capsaicin, a potent agonist of TRPV1. In TDI-sensitised wild-type mice, AHR was absent 24 h after instillation of AITC or capsaicin (fig. 6a and b; for individual responses see online supplementary fig. S2), despite evidence of successful sensitisation (online supplementary fig. S4), although *ex vivo* cytokine release was not increased in TDI/AITC and TDI/capsaicin mice (fig. 6d). Thus, it is not sufficient to activate TRPA1 or TRPV1 to induce AHR in TDI-sensitised mice.

Conversely, to test if the development of AHR necessitates an antigen-specific challenge, we gave TDI-sensitised mice a first challenge with AITC (nonspecific antigen) followed 1 week later by a second challenge either with TDI (specific antigen) or again with AITC. AHR was only induced after the challenge with TDI, *i.e.* in the TDI/AITC/TDI mice (fig. 6c; for individual responses see online supplementary fig. S2). All TDI-sensitised mice showed humoral (IgE) and cellular (T- and B- lymphocytes) evidence of immune sensitisation (online supplementary fig. S4), although the increased *ex vivo* release of IL-13, IFN- γ and IL-10 occurred in TDI/AITC/TDI mice and not in TDI/AITC/AITC mice, suggesting that the cytokine production depends on a challenge with the specific sensitiser (fig. 6d).

Involvement of immune cells in TDI-induced AHR

Taken together, the above results allowed us to conclude, first, that TRPA and TRPV1 are necessary, but not sufficient, to induce AHR in TDI-sensitised animals and, second, that a specific adaptive immune response is involved and necessary to induce AHR in such TDI-sensitised animals. Previous research on our mouse model has shown that lymphocytes are crucially involved in the development of TDI-induced

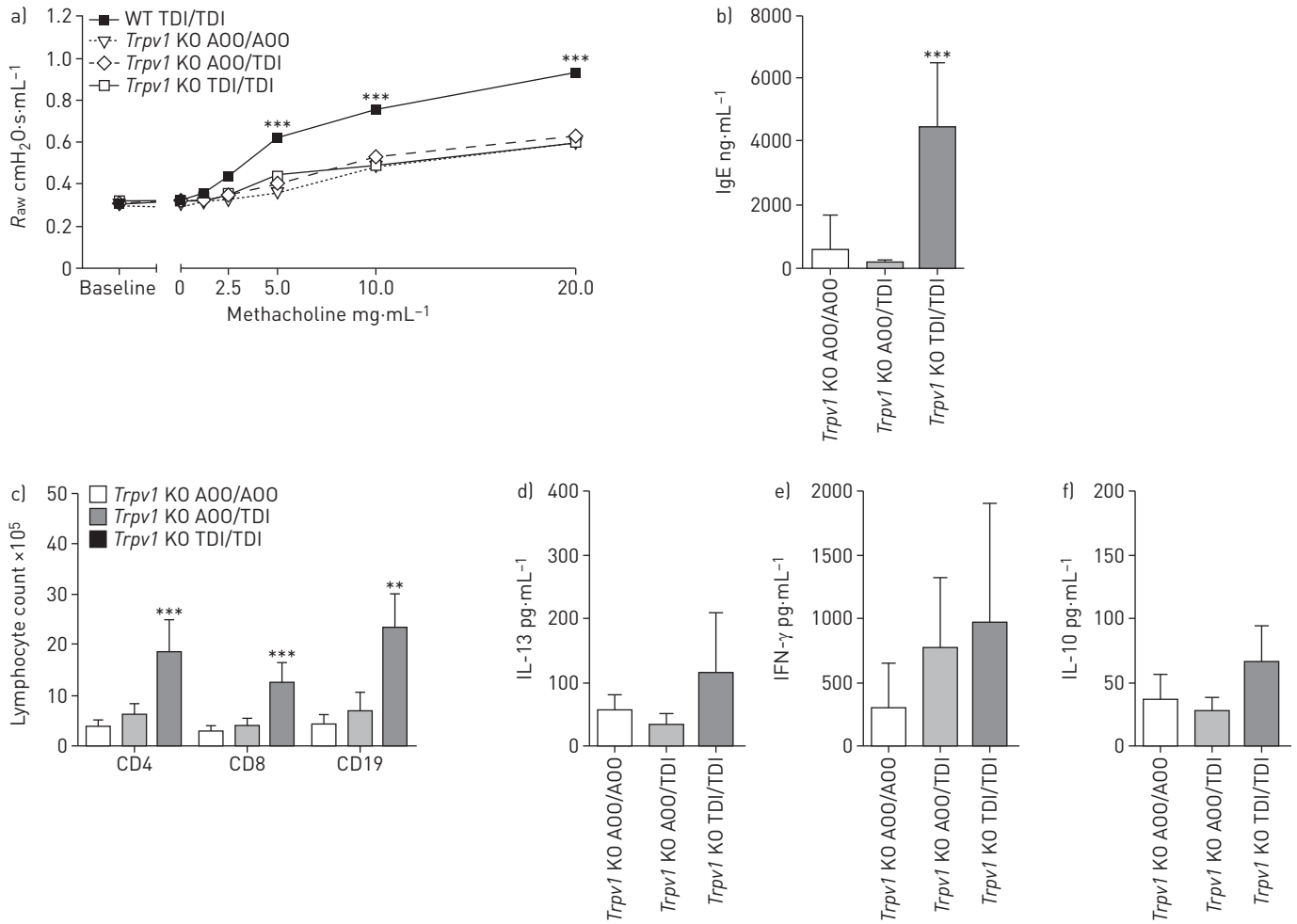


FIGURE 4 Involvement of transient receptor potential V1 (TRPV1) channels in toluene-2,4-diisocyanate (TDI)-induced airway hyperresponsiveness: *Trpv1* knockout (KO) mice. a) Airway resistance (R_{aw}) in response to methacholine in *Trpv1* KO mice; b) total serum IgE levels; c) number of T-helper cells (CD4⁺), cytotoxic T-cells (CD8⁺) and B-lymphocytes (CD19⁺); cytokine release of d) interleukin (IL)-13, e) interferon (IFN)- γ and f) IL-10. Data are presented as mean \pm SD, n=5-6 per group. WT: wild-type; AOO: acetone/olive oil. **: p<0.01; ***: p<0.001 compared to *Trpv1* KO AOO/AOO control group.

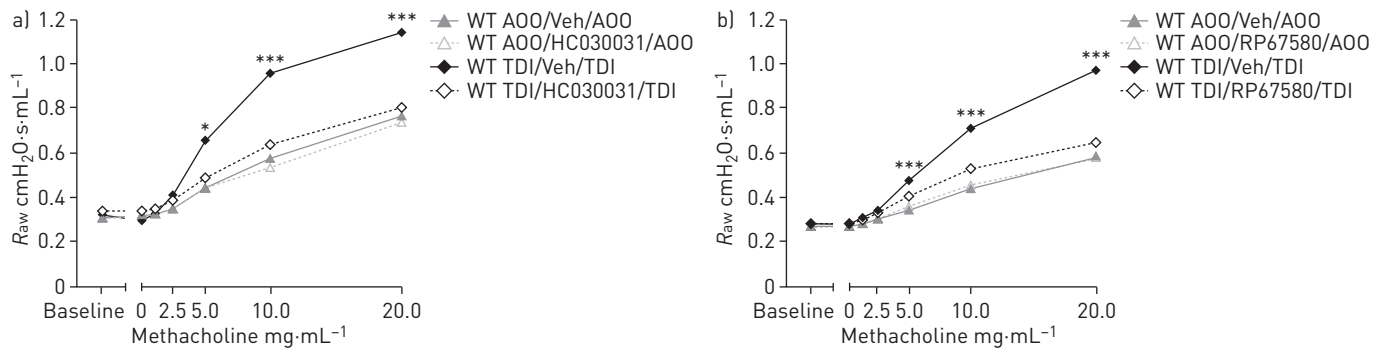


FIGURE 5 Involvement of transient receptor potential A1 (TRPA1) channels in toluene-2,4-diisocyanate (TDI)-induced airway hyperresponsiveness: pharmacological blockade. Airway resistance (R_{aw}) in response to methacholine in wild-type (WT) mice treated with a) either TRPA1 antagonist or vehicle (Veh) or b) either neurokinin 1 receptor antagonist or vehicle. AOO: acetone/olive oil. n=6-9 per group. *: p<0.5; ***: p<0.001 compared to corresponding wild-type AOO/Veh/AOO control group.

asthma in Balb/c mice [33, 34]. To determine whether this is also the case in the current model of chemical-induced AHR, using C57Bl/6 mice, we used *Rag2* knockout mice, lacking mature lymphocytes. Indeed, AHR was absent 24 h after instillation of TDI in TDI-sensitised *Rag2* knockout mice (online supplementary fig. S5a; for individual responses see online supplementary fig. S2). As expected, IgE levels were not increased in these mice (online supplementary fig. S5b).

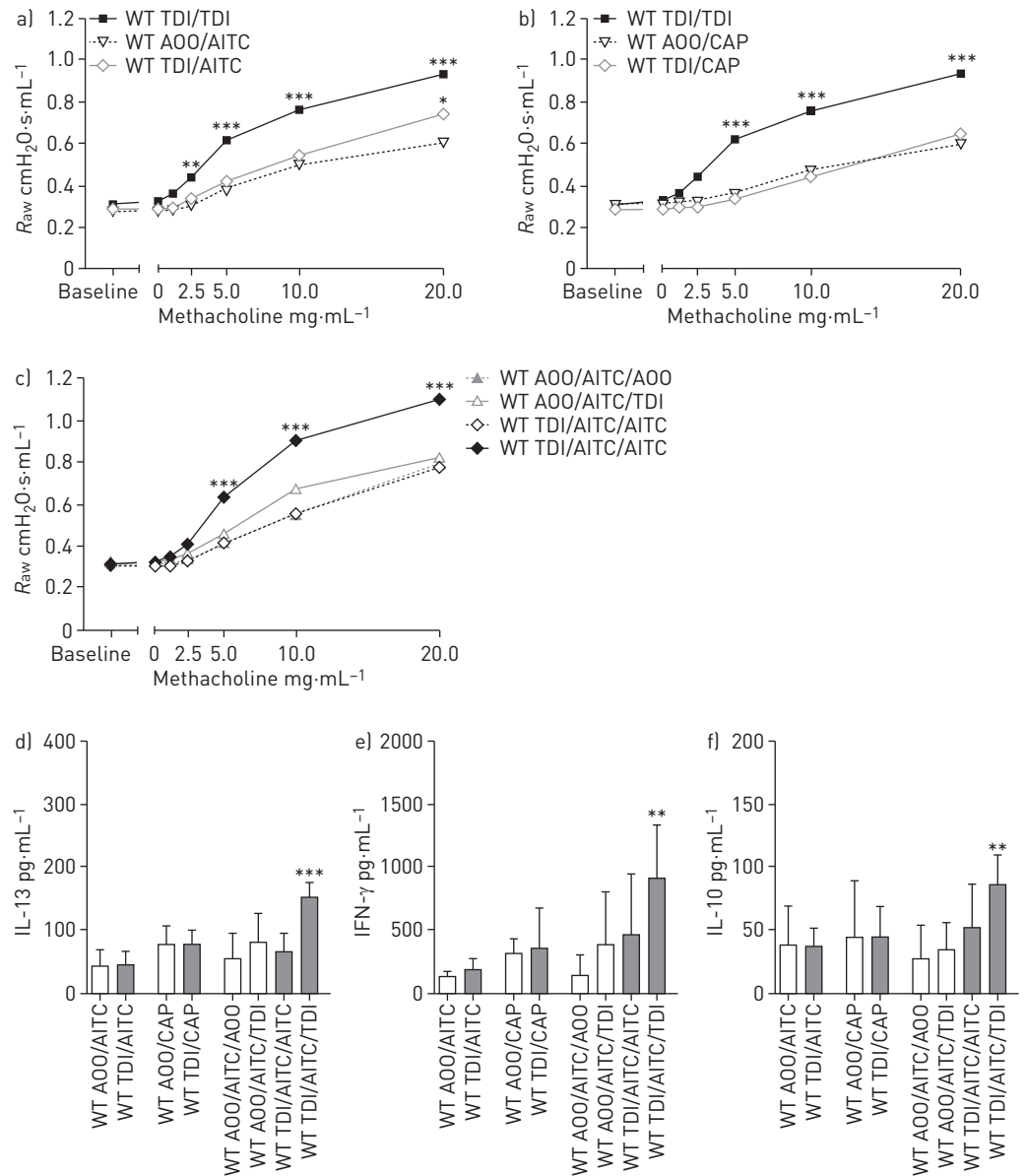


FIGURE 6 Induction of airway hyperresponsiveness depends on an allergen-specific challenge. Airway resistance (R_{aw}) in response to methacholine in wild-type (WT) mice after a first challenge with a) allyl isothiocyanate (AITC) or b) capsaicin (CAP) and c) after a second challenge with either acetone/olive oil (AOO), AITC or toluene-2,4-diisocyanate (TDI); cytokine release of d) interleukin (IL)-13, e) interferon (IFN)- γ and f) IL-10. Data are presented as mean \pm SD, $n=5-8$ per group. **: $p<0.01$; ***: $p<0.001$ compared to corresponding WT control group [AOO/AITC, AOO/CAP or AOO/AITC/AOO].

Since neurons have been proposed to directly communicate with mast cells [25], we also assessed the role of the latter in our model of chemical-induced AHR, by testing mast cell deficient mice ($Kit^{Wsh/Wsh}$ mice). In contrast to wild-type mice, no induction of AHR was observed in TDI-sensitised and TDI-challenged $Kit^{Wsh/Wsh}$ mice, even though they were responsive to methacholine (fig. 7a; for individual responses see online supplementary fig. S2). TDI-sensitised and TDI-challenged mast cell knockout mice showed the same humoral, cellular and *ex vivo* changes indicative of sensitisation as similarly treated wild-type mice (fig. 7b-d). To further validate the involvement of mast cells in TDI-induced AHR, wild-type mice were treated with the mast cell stabiliser ketotifen 30 min prior to the TDI challenge. In these TDI-sensitised mice, mast cell stabilisation abolished the induction of AHR 24 h after the TDI challenge (fig. 8; for individual responses see online supplementary fig. S2), while immune sensitisation was unaffected (online supplementary fig. S6). These results demonstrate that mast cells are critical for the development of AHR when TDI-sensitised mice are challenged with TDI.

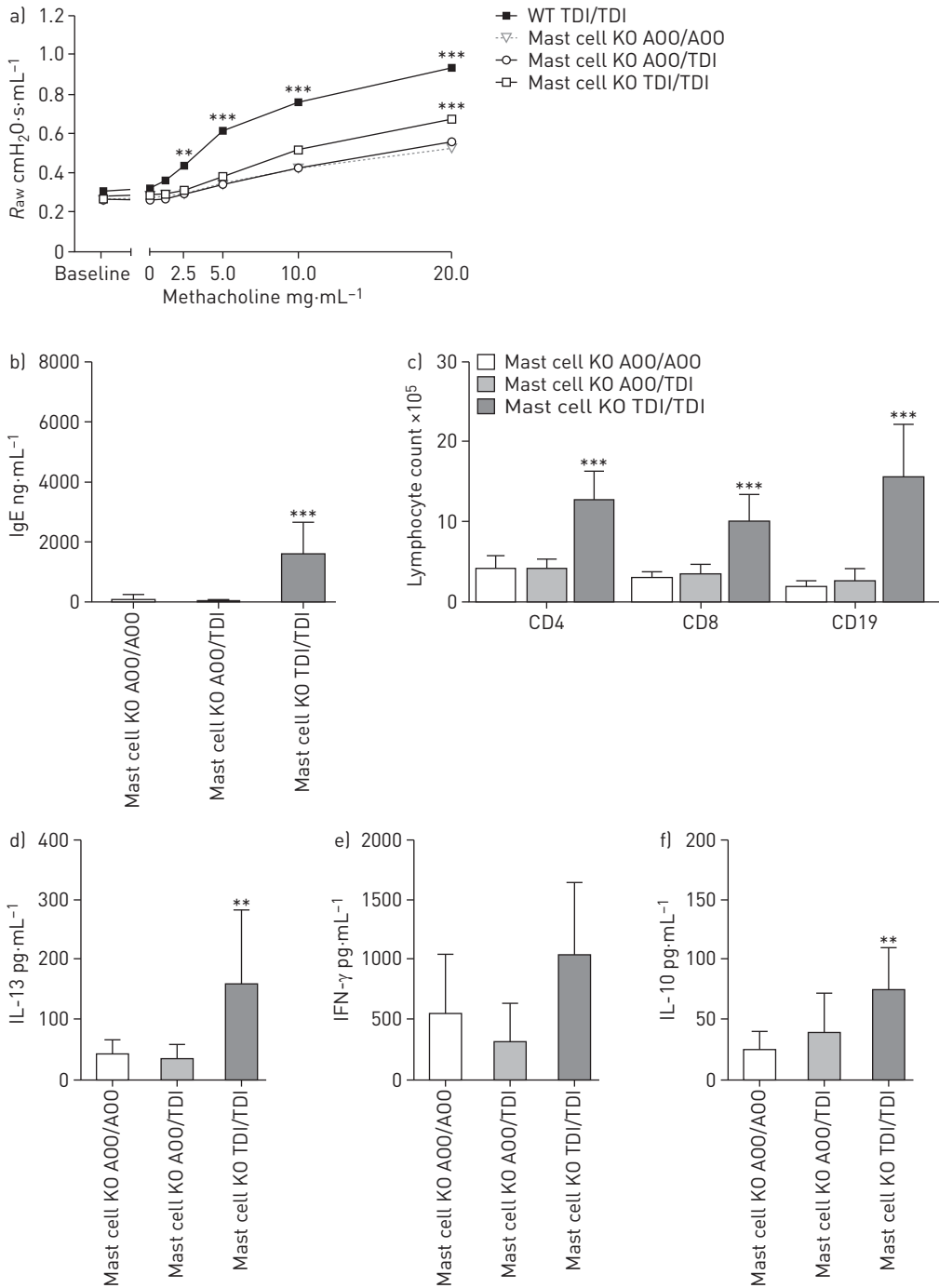


FIGURE 7 Involvement of mast cells in toluene-2,4-diisocyanate (TDI)-induced airway hyperresponsiveness: *Kit^{Wsh/Wsh}* mice. a) Airway resistance (R_{aw}) in response to methacholine in *Kit^{Wsh/Wsh}* mice; b) total serum IgE levels; c) number of T-helper cells (CD4⁺), cytotoxic T-cells (CD8⁺) and B-lymphocytes (CD19⁺); cytokine release of d) interleukin (IL)-13, e) interferon (IFN)- γ and f) IL-10. Data are presented as mean \pm SD, n=5–10 per group. WT: wild type; KO: knockout; A00: acetone/olive oil. **: p<0.01; ***: p<0.001 compared to corresponding mast cell KO (*Kit^{Wsh/Wsh}*) controls (A00/A00 or A00/vehicle/A00).

Discussion

To explore the interplay between neural and immunological mechanisms in asthma, we investigated the critical determinants of the respiratory responses to TDI, a chemical irritant and sensitiser that is known to cause immune-mediated asthma. We used a well-validated experimental model [11, 33].

The novelty of our work lies in the demonstration that TDI can directly activate mTRPA1, but not mTRPV1, and that, interestingly, both TRPA1 and TRPV1 are critical (although not sufficient) for the

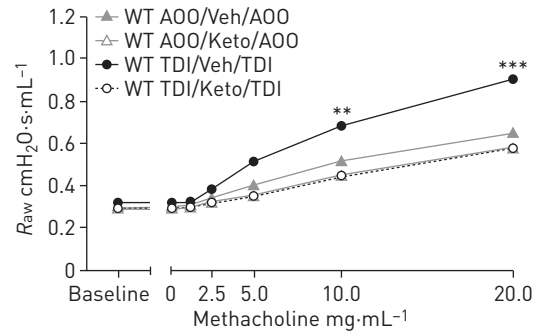


FIGURE 8 Involvement of mast cells in toluene-2,4-diisocyanate (TDI)- induced airway hyperresponsiveness: pharmacological blockade. Airway resistance (R_{aw}) in response to methacholine in wild-type (WT) mice treated with either ketotifen (Keto) or vehicle (Veh). $n=5-6$ per group. AOO: acetone/olive oil **: $p<0.01$; ***: $p<0.001$ compared to WT AOO/Veh/AOO control group.

induction of AHR in our model. We also established convincingly that mast cells are crucially involved in the induction of this AHR.

In our model of chemical-induced asthma, TDI-sensitised mice develop AHR 24 h after an intranasal TDI challenge. This response of the lower airways is indicative of the occurrence of a nasobronchial cross-talk, which is probably mediated by sensory nerves that are abundantly expressed in the upper airways. The existence of such cross-talk was demonstrated by HENS *et al.* [35] in an allergic OVA model in animals in which asthmatic responses were produced in spite of interruption of the continuity between the nose and lungs by means of a tracheostomy. The AHR that developed rapidly in these tracheostomised OVA-sensitised mice after a nasal challenge with OVA was attributed to pulmonary upregulation of substance P and activation of the NK1 receptor; hence neurogenic lung inflammation originating in the nose [35]. Thus, both the findings of HENS *et al.*, who used a model of atopic asthma, and our present observations in a model of chemical-induced asthma, support the concept of the “united airways”, from the nose to the lungs. Furthermore, our study provides novel mechanistic insight by demonstrating that the cross-talk operates through activation of TRPA1 and TRPV1 channels and with involvement of mast cells.

Using *in vitro* intracellular Ca^{2+} imaging, we showed channel activity in mTRPA1-expressing cells, but not in mTRPV1-expressing cells, in response to TDI. Direct evidence for the implication of TRPA1 was then obtained using electrophysiology studies, which were not performed for TRPV1 due to its unresponsiveness to TDI in the Ca^{2+} imaging experiments. Importantly, a previous study suggested that TDI activates TRPA1 [36], but this contention was based on Ca^{2+} imaging experiments, which provide only indirect indication of channel activity. Here, we used patch-clamp recordings and demonstrated, for the first time, that TDI activates TRPA1 channels and that this activation needs the presence of the isocyanate moieties, since TDA (having amine groups) was inactive.

Complementary to the *in vitro* studies, *in vivo* respiratory physiology experiments were performed, using the flexiVent to assess changes in nonspecific airway reactivity to methacholine, 1 day after the intranasal instillation [30]. The principal breakthrough that emerges from our studies is that both TRPA1 and TRPV1 are essential for the development of AHR after a challenge with TDI in TDI-sensitised mice. Our findings in *Trpa1* and *Trpv1* knockout mice and the validation using pharmacological agents, a TRPA1 antagonist or a NK1R antagonist, convincingly prove the involvement of TRPA1, TRPV1 and the neuropeptide substance P in immune-mediated chemical-induced AHR. Importantly, our experiments with TRP agonists (AITC and capsaicin) given to TDI-sensitised mice demonstrate that induction of AHR only occurred in a context of specific immune sensitisation, since AHR was only induced if the animals were challenged with the agent to which they had been sensitised. An involvement of TRPA1 in allergen-induced leukocyte infiltration and AHR has been reported, albeit without much mechanistic explanation, in a murine OVA model [37]. Moreover, in contrast with the latter study, we have used a model without cellular influx in the airways. Therefore, we were able to simulate paucigranulocytic asthma, a relevant condition that has hitherto hardly been studied. Furthermore, our model allowed us to disentangle the airway reactivity response from the immune-mediated inflammatory changes.

In addition, TRPV1 has been proposed to play a role in the development of allergic asthma [38, 39], although this was disputed by some others [37, 40]. In the current study, we show that TRPV1 is not activated directly by TDI, but is essential for the induction of AHR. However, the importance of TRPV1 activation can be attributed to its immunomodulatory function. Recently, TRPV1 has been demonstrated to regulate the activation and inflammatory properties of $CD4^{+}$ T-cells [41]. Furthermore, it has been

reported that IL-13, a key cytokine in AHR development, increases the level of TRPV1 in the murine lung and can induce increased TRPV1 expression in the bronchial epithelium of mice, linking TRPV1 to IL-13 induced features of asthma. Indeed, inhibition of TRPV1 has shown to attenuate IL-13 induced AHR, airway inflammation, goblet cell metaplasia and subepithelial fibrosis [38]. As such, we hypothesise that IL-13, released by lymphocytes or mast cells, can stimulate TRPV1. Previously we have described the importance of CD4⁺ T-cells and the Th2-cytokine IL-13 in our model of chemical-induced asthma, which could thus explain why AHR is abolished in TRPV1-deficient mice [33].

Furthermore, it has been shown that inflammatory mediators, such as prostaglandins, leukotrienes and bradykinin released by mast cells can reduce the activation threshold of TRPV1 [42, 43], and that neuropeptides released from sensory nerves can sensitise TRPV1 and enhance afferent excitability [44]. The inflammatory conditions can thus contribute to the activation and involvement of TRPV1 in our model.

In our search for the mechanism of enhanced nonspecific airway reactivity shortly after an antigen-specific challenge we also focused on mast cells, because of their prominent role in asthma [45] and because of the postulated interplay between mast cells and sensory neurons [46]. Our findings with mast cell deficient mice (*Kit^{Wsh/Wsh}* mice), validated by experiments using a mast cell stabiliser, demonstrate that functional mast cells are essential for the development of chemical-induced AHR after a TDI challenge in TDI-sensitised mice. We have previously reported the importance of mast cells in a model of nonallergic AHR [29], and this has also been shown in mouse models of atopic asthma [47, 48]. In the case of atopic asthma, activation of mast cells by allergen cross-linking of the IgE-bound high-affinity IgE receptor (FcεRI) leads to the release of various pro-inflammatory mediators that increase the sensitivity of airway smooth muscle to nonspecific stimuli, such as methacholine [45]. How mast cells become activated and which mediators they release in our model remain to be investigated. Based on previous evidence [49], a role for allergen cross-linking with IgEs seems unlikely. However, there is no doubt that the adaptive immune system is also involved, both because the induction of AHR relies on an antigen-specific challenge and because our model is dependent on properly functioning lymphocytes, as shown here with *Rag2* knockout mice and previously in severe combined immunodeficiency disease mice [11] and in adoptive lymphocyte transfer experiments [33, 34].

What we do know is that mast cells are located in the vicinity of nerve fibres present in the airways [50], and this close association with sensory nerves allows mast cells to respond immediately to neuropeptides released from nearby sensory nerve endings. SCHEERENS *et al.* [22] suggested that tachykinin neuropeptides, such as substance P, were involved in TDI-induced tracheal hyperreactivity as measured *ex vivo*, and they proposed, without giving evidence, that neurogenic inflammation resulted from the release of bioactive mediators by mast cells. Human and rodent mast cells do express neuropeptide receptors, including the substance P receptor NK1, and high concentrations of substance P induce mast cell degranulation [51]. In contrast, low concentrations of substance P were shown to prime mast cells by lowering the threshold for subsequent stimulation [52]. Conversely, mast cell activation and subsequent mediator release can sensitise afferent sensory fibres, by lowering their stimulation threshold [46]. In summary, various lines of evidence suggest the existence of bidirectional interaction pathways between mast cells and airway sensory nerves.

In conclusion, our results demonstrate that the induction of airway hyperreactivity by a chemical sensitiser such as TDI relies both on a specific adaptive immune response (that operates *via* lymphocyte activation) and on neuro-immune interactions, with crucial involvement of TRPA1, TRPV1 and mast cells. Our finding that TDI-induced asthma relies on neuro-immune mechanisms, involving both TRPA1, TRPV1 and mast cells, may lead to novel personalised strategies for treating patients with paucigranulocytic asthma. Patients with this phenotype of asthma may benefit from therapies targeting mast cell-TRP interactions, *via* the use of neuropeptide receptor blockers, mast cell stabilisers or TRPA1 blockers.

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