Eur Respir J 2008; 31: 773–782
DOI: 10.1183/09031936.00058907

Mast cell-derived tumour necrosis factor is essential for allergic airway disease

S. Reuter*, A. Heinz*, M. Sieren*, R. Wiewrodt*, E.W. Gelfand#, M. Stassen¶, R. Buhl* and C. Taube*,#

ABSTRACT: Mast cells are thought to contribute to allergic airway disease. However, the role of mast cell-produced mediators, such as tumour necrosis factor (TNF), for the development of allergic airway disease is unclear.

In order to define the role of mast cells in acute allergic airway disease two strains of mast cell-deficient mice (Kit$^{W/Wv}$ and Kit$^{W\text{-}sh/W\text{-}sh}$) were studied.

Compared with their wild-type littermates, Kit$^{W/Wv}$ and Kit$^{W\text{-}sh/W\text{-}sh}$ mice developed significantly lower airway responsiveness to methacholine and less airway inflammation and goblet cell metaplasia, following sensitisation in the absence of adjuvant and airway challenge. Transfer of bone marrow-derived mast cells (BMMCs) from wild-type mice to Kit$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted both airway responsiveness and inflammation to levels similar to those in sensitised and challenged wild-type mice. In contrast, sensitised Kit$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with BMMCs from TNF-deficient mice were still severely impaired in their ability to develop airway hyperresponsiveness, inflammation or goblet cell metaplasia following allergen challenge.

The present results demonstrate the significance of mast cells in the development of airway disease and highlight the importance of mast cell-derived tumour necrosis factor in these responses.

KEYWORDS: Asthma, immunology, mast cell, tumour necrosis factor

Asthma is a complex syndrome characterised by airway hyperresponsiveness (AHR), airway inflammation and airway obstruction [1]. An additional feature of allergic asthma is increased production of immunoglobulin (Ig)E in response to common environmental allergens, and a relationship between atopy and allergic asthma has been demonstrated in several studies [2, 3]. Many inflammatory cells, principally mast cells predominantly located at the mucosal interface between host and environment, have been implicated in the allergic airway response and are regarded as important effector cells in the allergic immune response. This is mainly due to the allergen-specific activation of these cells through the IgE-loaded high-affinity IgE receptor (FcεRI) following contact with allergen. Indeed, increased numbers of mast cells have been found in human asthmatics in close proximity to airway smooth muscle suggesting a potential role for the development and maintenance of allergic airway disease [4].

Several studies in murine models [5–7] support potential roles for mast cells in allergic airway disease. Mast cells, following activation, are able to degranulate and produce a plethora of different mediators [8]. Some of those mediators have been implicated in the chemotaxis of T-cells that are important for the development of allergic airway disease [9–11]. One of the many pro-inflammatory cytokines is tumour necrosis factor (TNF), which can be pre-formed and stored in mast cells and released upon demand within minutes [12–14]. Interestingly, mast cell-derived TNF has been found to promote T-cell activation and proliferation [15, 16], as well as the migration of dendritic cells [17]. However, the role of mast cell-derived TNF for the development of allergic airway disease is not well described.

The aim of the present study was to investigate the role of mast cells and mast cell-produced TNF in the induction of allergic airway disease in a model of allergen sensitisation without adjuvant. It is shown herein that mast cell-deficient mice do not develop allergic airway disease following sensitisation in the absence of adjuvant and airway challenge. Additionally, the present authors demonstrate that TNF produced by mast

This article has supplementary material accessible from www.erj.ersjournals.com

AFFILIATIONS
*III. Medical Clinic, Dept of Pulmonary Medicine, and
¶Institute for Immunology, University of Mainz, Mainz, Germany.
#Division of Cell Biology, Dept of Paediatrics, National Jewish Medical and Research Center, Denver, CO, USA.

CORRESPONDENCE
C. Taube
III. Medical Clinic
Dept of Pulmonary Medicine
Johannes Gutenberg University
Langenbeckstr. 1
55101 Mainz
Germany
Fax: 49 6131176668
E-mail: taube@3-med.klinik.uni-mainz.de

Received:
May 15 2007
Accepted after revision:
December 12 2007

SUPPORT STATEMENT
The present study was funded by Deutsche Forschungsgemeinschaft (SFB 548, A11 to C. Taube, and A10 and STA984/1-1 to M. Stassen), NIH-grants HL-36577 and HL-61005, and EPA grant R825702 (all to E.W. Gelfand and MAIFOR).

STATEMENT OF INTEREST
None declared.

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

cells is essential for the development of AHR, airway inflammation and goblet cell metaplasia.

METHODS

Mice

WB/ReJ-W$^{/+}$ and C57Bl/6J-W$^{v/+}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and the mast cell-deficient c-kit mutant F1-generation mice WBB6F1-Kit$^{W/W-v}$ and the congenic WBB6F1-Kit$^{+/+}$ were bred in the Zentrale Tierzuchtanstalt of the Johannes Gutenberg University Medical Center (Mainz, Germany). In addition, mast cell-deficient C57Bl/6-Kit$^{W-sh/W-sh}$ mice and congenic C57Bl/6-Kit$^{+/+}$ wild-type littermates were obtained by intercrossing C57Bl/6-Kit$^{W-sh/+}$ mice kindly provided by M. Maurer (Charité, Berlin, Germany). C57Bl/6 mice deficient for TNF were obtained from K. Steinbrink (University of Mainz, Mainz, Germany). Mice were used at age 8–12 weeks. For reconstitution experiments mice were 14 weeks old at the time of sensitisation. Animal procedures were conducted in accordance with current institutional guidelines and performed according to the Helsinki convention for the use and care of animals.

Experimental protocols

Experimental groups consisted of three or four mice per group and each experiment was performed at least twice. Mice were sensitised by intraperitoneal (*i.p.*) injection of 100 μL of 20 μg ovalbumin (OVA; Sigma-Aldrich, St Louis, MO, USA) solution in phosphate buffered saline (PBS) on days 0 and 14. Mice were then challenged *via* the airways on days 28, 29 and 30, using nebulised OVA (1% (weight/volume) in PBS) with an ultrasonic nebuliser (NE-U17; Omron, Hoofdorp, the Netherlands).

Mast cell reconstitution

In order to obtain bone marrow-derived mast cells (BMMCs), bone marrow from C57Bl/6 mice was cultured for 4–5 weeks in Iscove's modified Dulbecco's medium (10% (volume/volume) foetal calf serum, 50 μM β-mercaptoethanol, 2 mM glutamine, 100 μg·mL^{-1} streptomycin, 100 U·mL^{-1} penicillin, 20 U·mL^{-1} m-interleukin-3 and 200 ng·mL^{-1} kit-ligand) as described previously [18]. Nonadherent cells were transferred to fresh culture plates every 2–3 days for a total of $\geq$21 days in order to remove adherent macrophages and fibroblasts. After 4 weeks of culture, >95% of nonadherent cells contained granules that stained positively with toluidine blue and >95% expressed c-Kit on their surface as determined by fluorescence-activated cell sorting analysis using anti-c-Kit m-antibody. In order to reconstitute the mast cell-deficient mice (6-weeks-old C57Bl/6-Kit$^{Wsh/Wsh}$), 5x10^6 BMMCs were injected in the tail vein of each mouse. Sensitisation was started 8 weeks after the injection and airway challenges were performed at 12 weeks following BMMCs administration.

Measurement of airway reactivity

Measurements of airway resistance (R_L) were performed on anaesthetised, intubated and mechanically ventilated (FlexiVent; Scireq, Montreal, QC, Canada) mice in response to increasing doses of inhaled methacholine (MCh; 6.25, 12.5, 25, 50 and 100 mg·mL^{-1}). Measurements of R_L were performed every 15 s following each nebulisation step until a plateau phase was reached.

Bronchoalveolar lavage

After assessment of airway function, lungs were lavaged *via* the tracheal tube with PBS (1 mL). Numbers of lavaged cells were counted using trypan blue dye exclusion. Differential cell counts were made from cytocentrifuged preparations fixed and stained with a Microscopy Hemacolor®-Set (Merck, Darmstadt, Germany). Percentage and absolute numbers of each cell type were calculated. The numbers of CD3-, CD4- and CD8-positive cells was assessed by flow cytometry analysis using fluorescein isothiocyanate-conjugated monoclonal rat anti-mouse CD3 and phycoerythrin-conjugated rat anti-mouse CD4 or CD8 (all BD Bioscience Heidelberg, Germany). Absolute numbers of CD4-positive (CD3+/CD4+) and CD8-positive (CD3+/CD8+) T-cells were calculated by multiplying the total cell count and the percentage of either CD3/CD4 or CD3/CD8 cells.

Histology

Lungs were fixed by inflation (1 mL) and immersion in 10% (v/v) formalin, and embedded in paraffin. Tissue sections were stained with haematoxylin and eosin (HE) and periodic acid-Schiff (PAS). In order to assess reconstitution efficiencies, sections were used for fluorescence staining of tissue specific for mast cells with Avidin-Alexa-488 (Molecular probes; Invitrogen, Karlsruhe, Germany) [19–21] or were stained with toluidine blue. Slides were examined in a blinded fashion with a microscope (BX40; Olympus, Hamburg, Germany). The number of mast cells and goblet cells were analysed respectively using Avidin-Alexa-488 and PAS-stained slides and imaging software (Analysis; Soft Imaging Systems, Stuttgart, Germany). For the assessment for mast cell numbers in each slide, mast cells were counted by a blinded investigator in five different fields and, in each field, the lung area was measured using an image analysis system. Numbers of mast cells are expressed as cells·cm^{-2} [6, 22] and numbers of goblet cells are expressed as cells·mm^{-1} basement membrane (BM).

Antigen-specific ELISA

Serum was obtained 48 h following the last challenge. OVA specific IgG1 and IgG2b titres were determined using ELISA. Biotin-conjugated detection antibodies, streptavidin-horseradish peroxidase and substrat-reagent (BD-Pharmingen, Heidelberg, Germany), were used in concentrations recommended by the manufacturer. OVA-specific IgE was assessed using a method described by SPERGEL *et al.* [23]. Briefly, plates were coated with rat anti-mouse IgE (clone R35-72; BD-Pharmingen). Following administration of 3% (v/v) bovine serum albumin-PBS for 2 h, serial dilutions of sera were incubated overnight at 4°C. Then, biotin-labelled OVA was added for 2 h and absorption was read after addition of streptavidin-horseradish peroxidase and *o*-phenylenediamine. The antibody titre was defined as the reciprocal serum dilution yielding an optical density, measured at 450 nm, of 0.2 after linear regression analysis.

Statistical analysis

ANOVA was used to determine the levels of difference between all groups. Comparisons for all pairs were performed by the Tukey–Kramer honest significant difference test. A p-value <0.05 was considered to be significant. Values for all measurements are expressed as mean$\pm$SEM.

RESULTS

Mast cells are not required for allergen-specific B-cell and T-cell responses

In order to assess the role of mast cells following sensitisation without adjuvant, two different strains of mast cell-deficient mice were sensitised with OVA and then challenged with inhaled OVA on three consecutive days. Following sensitisation and challenge, WBB6F1-Kit$^{W/Wv}$ and C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice showed an increase in OVA-specific serum IgG1 and IgE titres, similarly to sensitised and challenged wild-type (WBB6F1-Kit$^{+/+}$ and C57Bl/6-Kit$^{+/+}$, respectively) mice (table 1). In addition, CD4+ T-cells derived from the spleens of sensitised and challenged mast cell-deficient and wild-type mice displayed a comparable response with respect to T-helper type 2 cytokine production after re-stimulation *in vitro* (see table in supplementary data).

Mast cells are necessary for the development of allergic airway disease

In order to investigate the role of mast cells in the development of AHR, lung function was assessed 48 h after the last allergen inhalation. Both strains of mast cell-deficient WBB6F1-Kit$^{W/Wv}$ mice and C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice showed no increase in airway responsiveness compared with respective wild-type mice, which displayed increased airway responsiveness to MCh following sensitisation and challenge (fig. 1). As shown in figure 2a, wild-type mice developed an eosinophilia in the airways, whereas in mast cell-deficient mice only few eosinophils appeared in the bronchoalveolar lavage (BAL) fluid. Additionally, numbers of CD4+ and CD8+ T-cells in BAL fluid were significantly lower in sensitised and challenged C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice compared with wild-type mice (fig. 2b). A reduction in tissue inflammation was also evident in HE-stained sections of lung tissue derived from mast cell-deficient mice compared with their congenic littermates after sensitisation and challenge (fig. 3a, d, g and j). Using PAS staining (fig. 3b, c, e, f, h, i, k and l), significantly reduced numbers ($p<0.01$) of goblet cells in airway epithelia of sensitised and challenged C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice were seen (27 ± 8 *versus* 93 ± 8 PAS-positive cells·mm^{-1} BM in C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ and wild-type mice, respectively; $p<0.01$, $n=12$). Using the same model, decreased airway inflammation, failure to develop AHR and reduced numbers of goblet cells were also confirmed using WBB6F1-Kit$^{W/Wv}$ animals, a different mouse strain that is also devoid of mast cells (data not shown).

Mast cell-derived TNF is pivotal for the development of AHR

Previous studies have shown that mast cell function can be restored in mast cell-deficient mice by transfer of BMMCs [5, 6]. Taking advantage of this technique, the role of mast cell-derived TNF was examined in the present model of acute allergic airway disease. To this end, BMMCs were obtained from either C57Bl/6-TNF$^{+/+}$ or C57Bl/6-TNF$^{-/-}$ donors. Following cross-linking of FcεRI *in vitro*, no differences in degranulation and production of cytokines (IL-4, -6 and -13) were found between the TNF-deficient mast cells and wild-type mast cells except for the production of TNF, which was absent in the BMMCs from C57Bl/6-TNF$^{-/-}$ donors (data not shown).

TABLE 1 Serum immunoglobulin titres

	WBB6F1-Kit$^{+/+}$		WBB6F1-Kit$^{W/Wv}$		C57Bl/6-Kit$^{+/+}$		C57Bl/6-Kit$^{Wsh/Wsh}$	
	Challenged	Sensitised and challenged	Challenged	Sensitised and challenged	Challenged	Sensitised and challenged	Challenged	Sensitised and challenged
OVA specific IgE	ND	352.7±64.4#	ND	315.2±57.9#	ND	42.3±12.7*	ND	39.5±12.1*
OVA specific IgG1 ×10^3	ND	117.7±25.5#	ND	90.6±28.7#	ND	45.7±11*	ND	38.3±19*
OVA specific IgG2b	18±4	1393±287#	25±7	1412±402#	19±4	584±117*	15±3	426±119*

Data are presented as mean±SEM. Serum levels of immunoglobulins (Ig) were assessed 48 h after the last challenge. WBB6F1-Kit$^{+/+}$: congenic wild-type control mice; WBB6F1-Kit$^{W/Wv}$: mast cell-deficient mice; C57Bl/6-Kit$^{+/+}$ congenic wild-type controls; C57Bl/6-Kit$^{Wsh/Wsh}$: mast cell-deficient mice; ND: not detectable. #: $p<0.05$ compared with WBB6F1-Kit$^{+/+}$ challenged and WBB6F1-Kit$^{W/Wv}$ challenged. OVA: ovalbumin; *: $p<0.05$ compared with C57Bl/6-Kit$^{+/+}$ challenged and C57Bl/6-Kit$^{Wsh/Wsh}$ challenged.

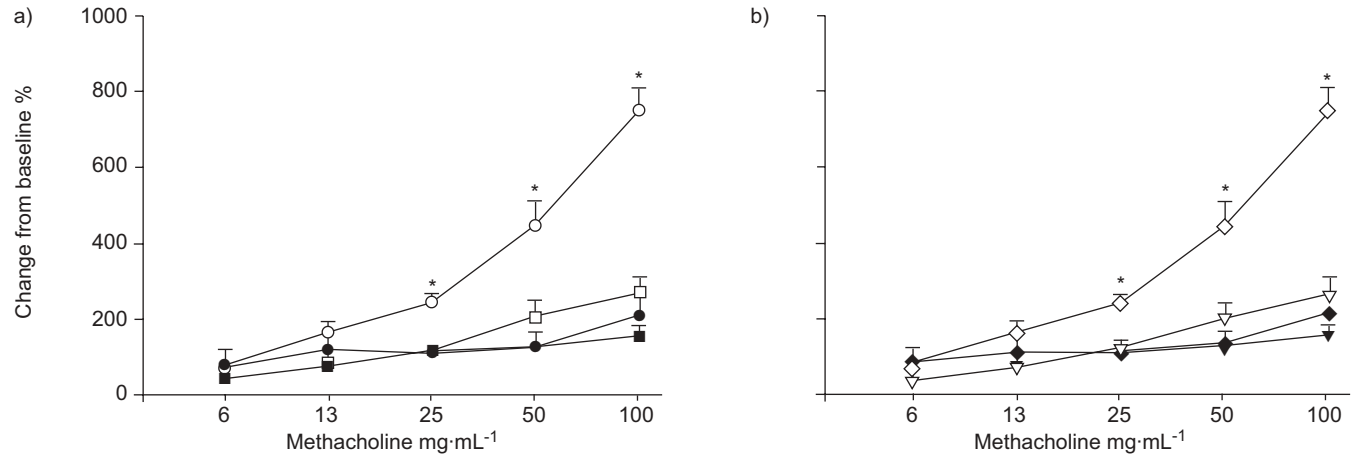

FIGURE 1. Airway responsiveness in mast cell-deficient mice. a) Airway responsiveness (resistance) in challenged only (■; n=10) and sensitised and challenged (□; n=10) WBB6F1-Kit$^{W/W-v}$ mice, and in challenged only (●; n=10) and sensitised and challenged (○; n=10) wild-type (WBB6F1-Kit$^{+/+}$) mice. b) Airway responsiveness in challenged only (▼; n=12) and sensitised and challenged (▽; n=12) C57Bl/6-Kit$^{W-sh/W-sh}$ mice, and in challenged only (◆; n=12) and sensitised and challenged (◇; n=12) wild-type (C57Bl/6-Kit$^{+/+}$) mice. Data are shown as mean±SEM. *: $p<0.05$ compared with all other groups.

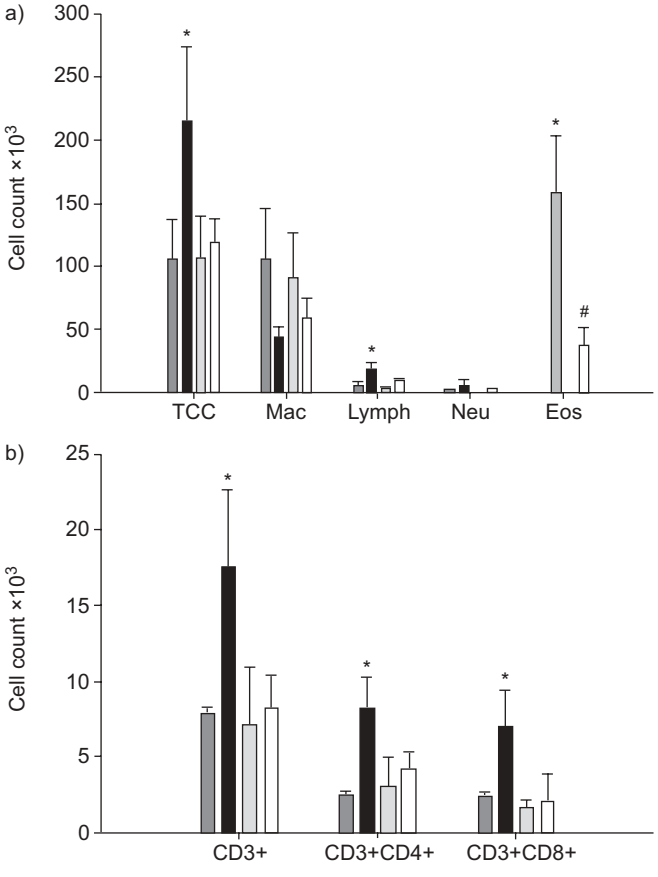

FIGURE 2. Airway inflammation in bronchoalveolar lavage (BAL) fluid. a) Differential cell count and b) number of CD4+ and CD8+ T-cells were assessed in BAL fluid of challenged only (■; n=12) and sensitised and challenged C57Bl/6-Kit$^{+/+}$ (■; n=12) mice, and challenged only (■; n=12) and sensitised and challenged C57Bl/6-Kit$^{W-sh/W-sh}$ (□; n=12) mice. Data are shown as mean±SEM. TCC: total cell count; Mac: macrophages; Lymph: lymphocytes; Neu: neutrophils; Eos: eosinophils. *: $p<0.05$ compared with all other groups; #: $p<0.05$ compared with C57Bl/6-Kit$^{+/+}$ challenged only and C57Bl/6-Kit$^{W-sh/W-sh}$ challenged-only mice.

BMMCs derived from C57Bl/6-TNF$^{+/+}$ or C57Bl/6-TNF$^{-/-}$ donors were transferred to C57Bl/6-Kit$^{W-sh/W-sh}$ recipient mice. Recipient mice were sensitised and challenged 8 weeks after reconstitution. In mice reconstituted with mast cells from C57Bl/6-TNF$^{+/+}$ animals (n=9), 35±12 mast cells·cm^{-2} were detected compared with 20±5 cells·cm^{-2} following reconstitution with mast cells from C57Bl/6-TNF$^{-/-}$ donors (n=9; $p>0.05$ compared to all other groups) and 30±12 cells·cm^{-2} in sensitised and challenged wild-type mice (n=9; fig. 4 and figure in supplementary data).

C57Bl/6-Kit$^{W-sh/W-sh}$ mice reconstituted with wild-type BMMCs showed airway responsiveness similar to sensitised and challenged wild-type mice (fig. 5). In contrast, reconstitution of mast cell-deficient mice with BMMCs derived from C57Bl/6-TNF$^{-/-}$ donors did not restore AHR (fig. 5). This clearly indicates an important role of mast cell-derived TNF for the development of AHR.

Airway inflammation and goblet cell metaplasia in mast cell-deficient mice cannot be restored upon transfer of TNF-deficient mast cells

Using reconstitution experiments, the influence of mast cells and mast cell-derived TNF on airway inflammation and goblet cell hyperplasia was analysed. As summarised in figures 4 and 6, numbers of eosinophils and T-cells in BAL (fig. 6), as well as cellular infiltration in the lungs (figs 4a–e) and number of goblet cells (figs 4f–j and table 2), were restored to wild-type levels upon transfer of wild-type BMMCs into mast cell-deficient mice. However, reconstitution of mast cell-deficient mice with BMMCs derived from C57Bl/6-TNF$^{-/-}$ donors did not restore airway inflammation (figs 4 and 6) and goblet cell metaplasia (fig. 4 and table 2).

DISCUSSION

Several lines of evidence support a pivotal role of mast cells for the development of allergic asthma, but currently very little is known about the underlying mechanisms and mediators involved in these mast cell-dependent responses. In the

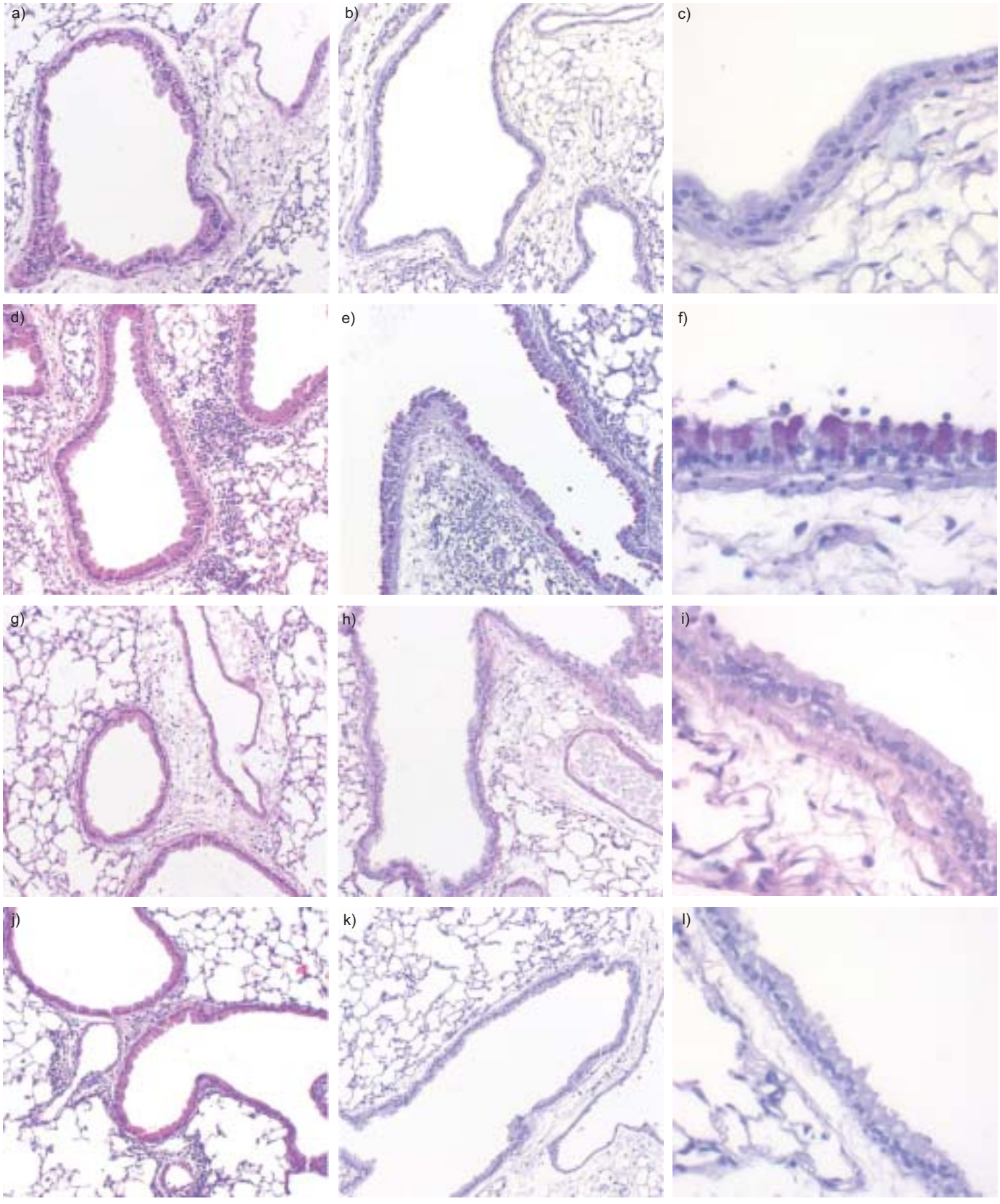

FIGURE 3. Tissue inflammation and goblet cell metaplasia in mast cell-deficient mice. Tissue inflammation was evaluated 48 h after the last challenge using haematoxylin and eosin staining (a, d, g and j) and periodic acid-Schiff staining (b, c, e, f, h, i, k and l) for goblet cells in challenged-only C57Bl/6-Kit$^{+/+}$ (a–c), sensitised and challenged C57Bl/6-Kit$^{+/+}$ (d–f), challenged-only C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ (g–i) and sensitised and challenged C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ (j–l) mice.

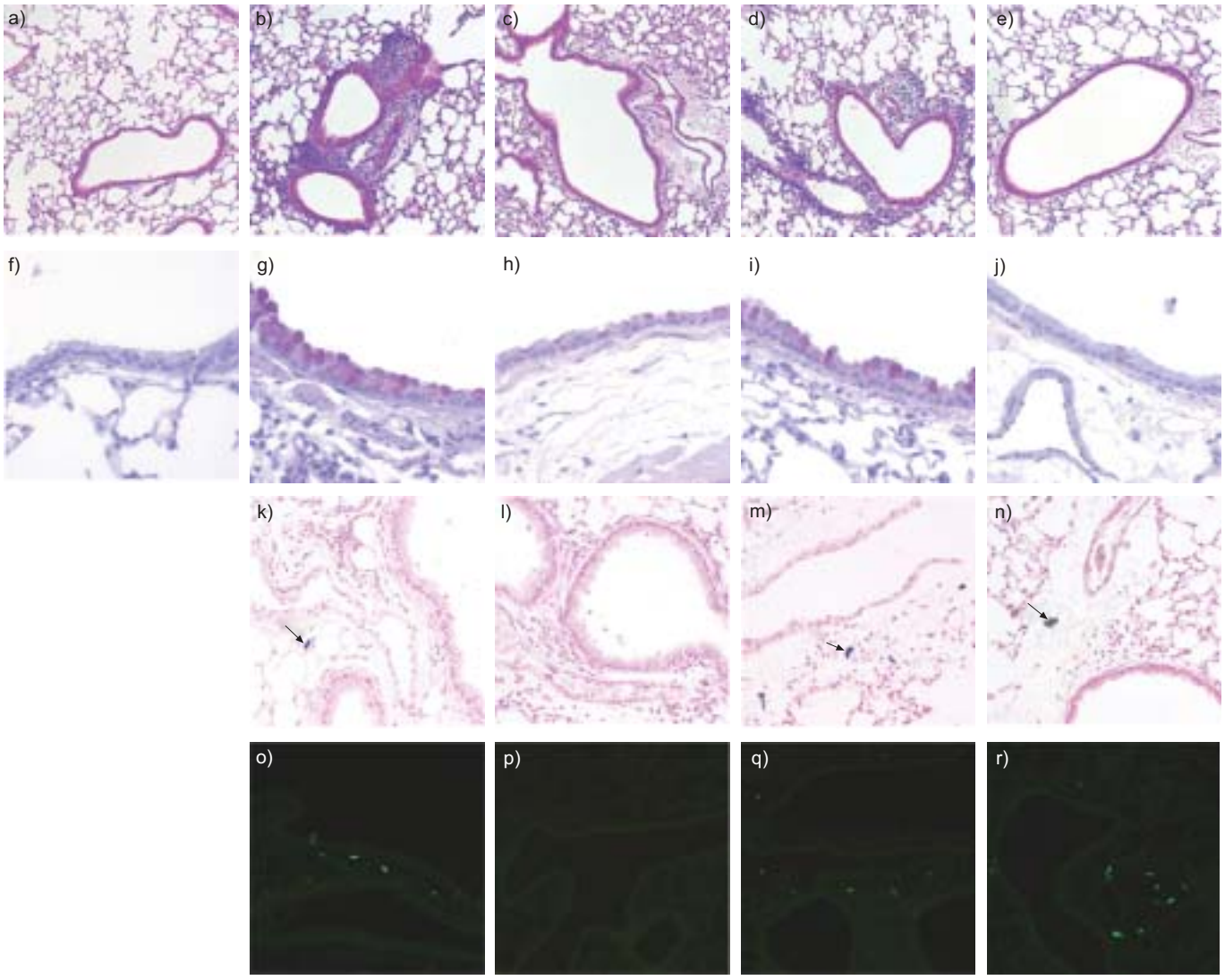

FIGURE 4. Tissue inflammation and goblet cell metaplasia in mast cell-deficient mice. Tissue inflammation was evaluated 48 h after the last challenge using haematoxylin and eosin staining (a–e), periodic acid-Schiff staining for goblet cells (f–j) and toluidine blue (k–n) and Avidin-Alexa-488 (o–r) staining for mast cells in challenged-only C57Bl/6-Kit$^{+/+}$ mice (a and f), sensitised and challenge C57Bl/6-Kit$^{+/+}$ mice (b, g, k and o), sensitised and challenge C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice (c, h, l and p), sensitised and challenge C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with bone marrow-derived mast cells (BMMCs) from wild-type mice (d, i, m and q) and sensitised and challenged C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with BMMCs from tumour necrosis factor-deficient mice (e, j, n and r). Arrows mark toluidine blue-stained cells.

present study, an acute model of allergic airway disease shows that, following sensitisation without adjuvant and challenge of the mice, mast cells are necessary for the development of AHR and airway inflammation. Furthermore, mast cell-derived TNF is shown to be an essential mediator for the development of these responses in the present model.

Mast cells have been postulated as important effector cells in allergic airway disease for a long time. However, the role and contribution of mast cells to the development of allergic airway disease appears to be highly dependent on the sensitisation and allergen exposure protocol in murine models. Studies using models of systemic sensitisation with adjuvant have repeatedly shown a similar degree of AHR and airway inflammation in mast cell- or IgE-deficient mice compared with respective wild-type mice [24–26]. In several different models with less potent sensitisation protocols, mast cells have been implicated to be necessary for the induction of nonallergic [27] but also allergen-induced airway disease [5–7, 28, 29]. In the present study, the authors show that following systemic allergen sensitisation without adjuvant and airway challenge, levels of allergen-specific IgE and IgG1 and allergen-induced proliferation of T-cells remained intact in both mast cell-deficient mouse strains. Also increased levels of OVA-specific IgG2b were detected. However, these were much lower compared with OVA-specific IgG1 levels, suggesting a predominant Th2 response in these animals. These findings and previous studies [6] suggest that, after systemic introduction of the allergen, sensitisation is not impaired in mast cell-deficient mice. In contrast, development of key features of allergic airway disease, including AHR, airway inflammation, migration of T-cells into the lung and goblet cell metaplasia,

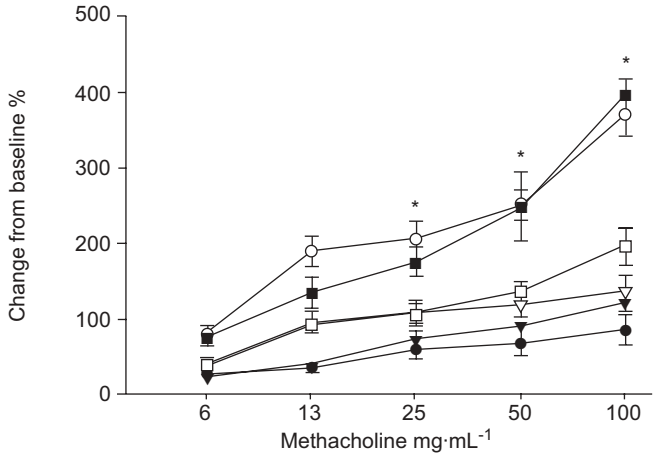

FIGURE 5. Airway responsiveness in mast cell-deficient mice following reconstitution with wild-type and tumour necrosis factor (TNF)-α deficient bone marrow-derived mast cells (BMMCs). Airway reactivity was assessed following sensitisation and challenge in C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ reconstituted with either BMMCs derived from wild-type mice (■; n=9) or TNF-deficient mice (□; n=9) and compared with challenged wild-type mice (●; n=9), sensitised and challenged wild-type mice (○; n=9), challenged C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice (▼; n=9) and sensitised and challenged C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice that were not reconstituted with mast cells (▽; n=9). Data are presented as mean±SEM. *: $p<0.05$ compared with C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ reconstituted with BMMCs derived from TNF-deficient, challenged-only C57Bl/6-Kit$^{+/+}$, challenged-only C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$, and sensitised and challenged C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice.

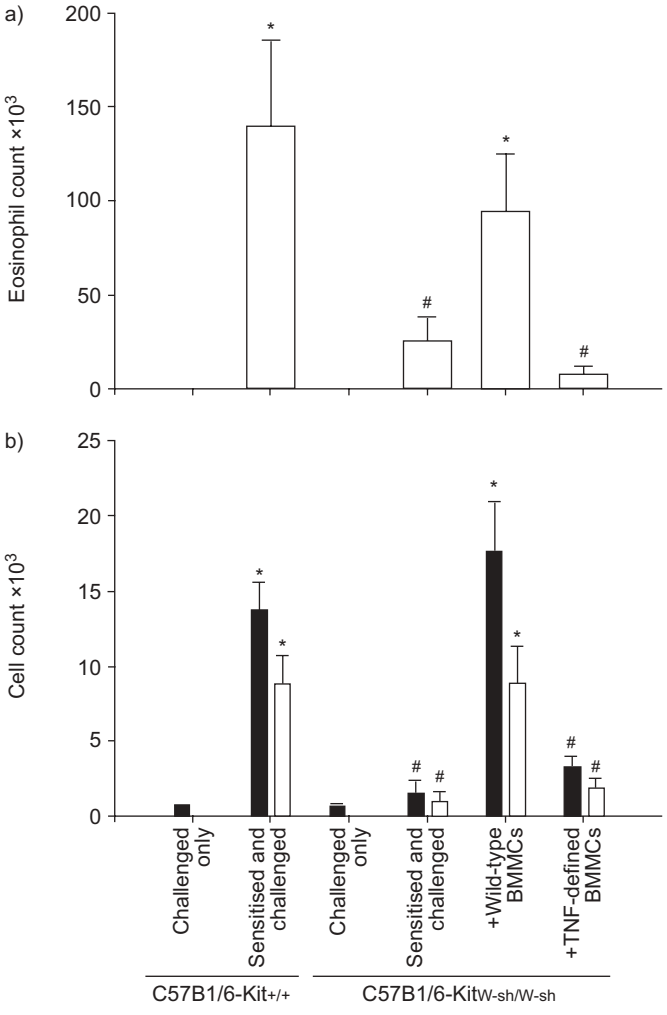

FIGURE 6. Eosinophil, CD4+ and CD8+ T-cell numbers in bronchoalveolar lavage (BAL) fluid. a) Number of eosinophils and b) number of CD4+ (■) and CD8+ (□) T-cells were assessed in the BAL of wild-type mice (C57Bl/6-Kit$^{+/+}$) which were challenged only or sensitised and challenged, and in C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice which were challenged only, sensitised and challenged and sensitised or challenged following reconstitution with bone marrow-derived mast cells (BMMCs) from wild-type (+wild-type BMMCs) or tumour necrosis factor (TNF)-deficient (TNF-deficient BMMCs) mice. Data are shown as mean±SEM from three independent experiments and n=9 for all conditions. *: $p<0.05$ compared with all other groups; #: $p<0.05$ compared with C57Bl/6-Kit$^{+/+}$ challenged-only and C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ challenged-only mice.

are decreased in both mast cell-deficient mouse strains, suggesting an important role for mast cells in mediating allergen-induced responses.

Transfer of BMMCs to mast cell-deficient mice has been shown to reconstitute mast cells in many organs but also the lungs of the recipient mice [22, 30]. Previous studies by WILLIAMS and GALLI [5] and YU *et al.* [6] in a more chronic model of allergic airway disease have also demonstrated that BMMC administration to mast cell-deficient mice reconstituted the features of allergic airway disease. Similarly, in the present study using a more acute model, transfer of BMMCs derived from C57Bl/6-Kit$^{+/+}$ mice to C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ recipients resulted in detectable mast cells in lung tissue accompanied by AHR, increased airway inflammation and goblet cell metaplasia in sensitised and challenged recipients to levels seen in sensitised and challenged wild-type mice.

Mast cells can be activated through different stimuli [31], primarily through IgE/allergen-mediated crosslinking of FcεRI in the context of allergic airway disease [32, 33]. YU *et al.* [6] showed that the expression of the Fc receptor for IgG (FcR γ-chain) in mast cells, which is necessary for the surface expression of the FcR γ-chains I and III and FcεRI receptor, is critical for the induction of most features of allergen induced lung pathology. Studies in a model of allergen inhalation have suggested that activation through FcεRI, which in mice is only displayed on mast cells and basophils [32], contributed to increased airway reactivity [7]. Despite several studies demonstrating a contribution of mast cells in the development of acute and chronic allergic airway disease, little is known about which mast cell-derived mediator(s) may be necessary for these responses. Following activation, mast cells are capable of producing a wide variety of mediators, including histamine, lipid mediators, chemokines and cytokines. Some of these mediators have been linked to the development of allergic airway disease, but it remains unclear whether mast cells are an essential source for them, as other cells types might also contribute to their production. Indeed, IL-13 has been repeatedly shown to be a central effector cytokine in allergic airway disease [34–36]. However, the production of IL-13 by mast cells does not seem to be necessary for the induction of increased airway reactivity following inhaled allergen challenge [7].

TABLE 2 Number of periodic acid-Schiff (PAS)-positive cells in airway epithelium

	C57Bl/6-Kit$^{+/+}$		C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$			
	Challenged only	Sensitised and challenged	Challenged only	Sensitised and challenged	WT BMMC	TNF BMMC
PAS-positive cells·mm^{-1} basement membrane	ND	65±11*	ND	20±11$^{\#}$	62±10*	21±9$^{\#}$

Data are presented as mean±SEM. C57Bl/6-Kit$^{+/+}$: congenic wild-type controls; C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$: mast cell-deficient mice; WT BMMC: reconstitution with bone marrow-derived mast cells (BMMCs) from wild-type donors; TNF BMMC: reconstitution with BMMCs from tumour necrosis factor (TNF)-deficient donors; ND: not detectable. *: $p<0.05$ compared with C57Bl/6-Kit$^{+/+}$ challenged-only, C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ challenged-only, C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ sensitised and challenged and C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ TNF BMMC; $^{\#}$: $p<0.05$ compared with C57Bl/6-Kit$^{+/+}$ challenged only and C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ challenged-only.

Conversely, specialised adoptive transfer models have suggested a role of mast cell-produced lipid mediators for recruitment of allergen-specific CD8+ T-effector cells into the lung [11], which are necessary for the development of allergen specific lung pathology [10]. In the present study, a decrease in CD4+ as well as CD8+ T-cells was detected in BAL fluid of the sensitised and challenged mast cell-deficient mice, suggesting impaired T-cell migration or local expansion in the lung consequent to the missing mast cell stimuli.

A mediator which has been implicated in many mast cell-dependent inflammatory responses is TNF. In agreement with the aforementioned reports, mast cell-deficient mice reconstituted with BMMCs from TNF-deficient donors in the present study failed to develop significant AHR following sensitisation and challenge. In addition, other features of allergic airway disease, such as airway inflammation and goblet cell metaplasia, were not reconstituted in mice engrafted with TNF-deficient mast cells, implicating a critical role of mast cell-derived TNF for the development of allergic airway disease. Mast cells have the capability to store and rapidly release TNF following activation [37, 38]. In other disease models, mast cell-derived TNF has been identified as being important for the induction and promotion of initial inflammatory events, *e.g.* in models of immune complex-induced inflammation [39], acute septic peritonitis [40], cutaneous inflammation [41], colitis [42], delayed-type hypersensitivity reactions [43] and pulmonary hypersensitivity reactions [44]. Several studies have also suggested a contribution of TNF during the induction of allergic airway disease. Following IgE-dependent stimulation of human lung tissue, TNF is produced in amounts sufficient to induce biological effects [45]. Additionally, administration of TNF into the lungs of mice induces increased airway mucus gene expression [46] and late airway response [47]. Recently, in TNF-deficient mice, a reduction in AHR and airway inflammation following a protocol of systemic sensitisation without an additional adjuvant and airway challenge was demonstrated [48]. Following allergen challenge, WBB6F1-Kit$^{W/Wv}$ mice expressed lower levels of TNF in BAL fluid and reconstitution with wild-type BMMCs restored BAL fluid levels of TNF and AHR [49]. Independently confirming the present results, NAKAE *et al.* [50] reported that mast cell-deficient mice reconstituted with BMMCs deficient in TNF failed to reconstitute the development of AHR. In a model of allergic airway disease different from the one used in the present experiments, it was shown that development of airway inflammation and goblet cell hyperplasia was impaired following reconstitution of C57Bl/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice with TNF-deficient BMMCs but not with wild-type BMMCs. The findings of the present study are in agreement with those results and extend them to a different model of allergic airway disease. The comparable results in two independent studies underscore the importance of mast cell-produced TNF. The clinical relevance of these latter findings was strengthened by recent studies in patients with moderate [51] and more severe asthma [52, 53], where the beneficial effects of treatment with TNF-neutralising antibodies were described. The fact that treatment with TNF-neutralising antibodies has shown promising first results in humans further supports the view that TNF is a potent therapeutic target for patients with allergic asthma.

The underlying mechanisms by which mast cell-derived TNF affects the different features of allergic airway disease remain to be elucidated. Based on current knowledge, it is conceivable that migration of dendritic cells from the lung to the regional lymph nodes is impaired in the presence of TNF-deficient mast cells [54]. This assumption is substantiated by reports showing that IgE-dependent mast cell activation induces Langerhans cell migration [55] and that mast cell-derived TNF directly influences dendritic cell migration from the lung [17]. However, a direct effect on T-cells, which are thought to orchestrate the allergic response [1], is just as conceivable, as TNF from mast cells can directly influence T-cell activation and proliferation [15, 16]. Indeed, NAKAE *et al.* [50] suggest an increase in T-cell activation triggered by mast cell-produced TNF as the main modulator for the development of AHR and airway inflammation.

In summary, the present study demonstrates a critical role of mast cells for the development of acute allergic airway disease following sensitisation without an adjuvant. This finding is in agreement with models of more chronic protocols [5, 6]. Based on the reconstitution experiments with tumour necrosis factor-deficient mast cells the pivotal role for mast cell-derived tumour necrosis factor in the allergen-induced development of

airway hyperresponsiveness, airway inflammation and goblet cell metaplasia after allergen exposure of the sensitised host was identified.

ACKNOWLEDGEMENTS

The authors would like to thank K. Steinbrink (Dept of Dermatology, University of Mainz, Mainz, Germany) for generously providing C57Bl/6-TNF-/- bone marrow, and Marcus Maurer (Dept of Dermatology, Charité, Berlin, Germany) for providing the C57Bl/6-Kit$^{W\text{-}sh/+}$ mouse strain.

REFERENCES

1 Busse WW, Lemanske RF Jr. Asthma. *N Engl J Med* 2001; 344: 350–362.
2 Burrows B, Martinez FD, Halonen M, Barbee RA, Cline MG. Association of asthma with serum IgE levels and skin-test reactivity to allergens. *N Engl J Med* 1989; 320: 271–277.
3 Peat JK, Tovey E, Toelle BG, *et al.* House dust mite allergens. A major risk factor for childhood asthma in Australia. *Am J Respir Crit Care Med* 1996; 153: 141–146.
4 Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med* 2002; 346: 1699–1705.
5 Williams CM, Galli SJ. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *J Exp Med* 2000; 192: 455–462.
6 Yu M, Tsai M, Tam SY, Jones C, Zehnder J, Galli SJ. Mast cells can promote the development of multiple features of chronic asthma in mice. *J Clin Invest* 2006; 116: 1633–1641.
7 Taube C, Wei X, Swasey CH, *et al.* Mast cells, FcεRI, and IL-13 are required for development of airway hyperresponsiveness after aerosolized allergen exposure in the absence of adjuvant. *J Immunol* 2004; 172: 6398–6406.
8 Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. *Nat Immunol* 2005; 6: 135–142.
9 Ott VL, Cambier JC, Kappler J, Marrack P, Swanson BJ. Mast cell-dependent migration of effector CD8+ T cells through production of leukotriene B4. *Nat Immunol* 2003; 4: 974–981.
10 Miyahara N, Swanson BJ, Takeda K, *et al.* Effector CD8+ T cells mediate inflammation and airway hyper-responsiveness. *Nat Med* 2004; 10: 865–869.
11 Taube C, Miyahara N, Ott V, *et al.* The leukotriene B4 receptor (BLT1) is required for effector CD8+ T cell-mediated, mast cell-dependent airway hyperresponsiveness. *J Immunol* 2006; 176: 3157–3164.
12 Gordon JR, Galli SJ. Mast cells as a source of both preformed and immunologically inducible TNF-α/cachectin. *Nature* 1990; 346: 274–276.
13 Echtenacher B, Männel DN, Hültner L. Critical protective role of mast cells in a model of acute septic peritonitis. *Nature* 1996; 381: 75–77.
14 Malaviya R, Ikeda T, Ross E, Abraham SN. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-α. *Nature* 1996; 381: 77–80.
15 Nakae S, Suto H, Kakurai M, Sedgwick JD, Tsai M, Galli SJ. Mast cells enhance T cell activation: Importance of mast cell-derived TNF. *Proc Natl Acad Sci USA* 2005; 102: 6467–6472.
16 Nakae S, Suto H, Iikura M, *et al.* Mast cells enhance T cell activation: importance of mast cell costimulatory molecules and secreted TNF. *J Immunol* 2006; 176: 2238–2248.
17 Suto H, Nakae S, Kakurai M, Sedgwick JD, Tsai M, Galli SJ. Mast cell-associated TNF promotes dendritic cell migration. *J Immunol* 2006; 176: 4102–4112.
18 Klein M, Klein-Hessling S, Palmetshofer A, *et al.* Specific and redundant roles for NFAT transcription factors in the expression of mast cell-derived cytokines. *J Immunol* 2006; 177: 6667–6674.
19 Bergstresser PR, Tigelaar RE, Tharp MD. Conjugated avidin identifies cutaneous rodent and human mast cells. *J Invest Dermatol* 1984; 83: 214–218.
20 Bussolati G, Gugliotta P. Nonspecific staining of mast cells by avidin-biotin-peroxidase complexes (ABC). *J Histochem Cytochem* 1983; 31: 1419–1421.
21 Heib V, Becker M, Warger T, *et al.*, *Mast cells are crucial for early inflammation, migration of Langerhans cells and CTL responses following topical application of TLR7 ligand in mice. Blood* 2007; 110: 946–953.
22 Wolters PJ, Mallen-St Clair J, Lewis CC, *et al.* Tissue-selective mast cell reconstitution and differential lung gene expression in mast cell-deficient Kit(W-sh)/Kit(W-sh) sash mice. *Clin Exp Allergy* 2005; 35: 82–88.
23 Spergel JM, Mizoguchi E, Brewer JP, Martin TR, Bhan AK, Geha RS. Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. *J Clin Invest* 1998; 101: 1614–1622.
24 Mehlhop PD, van de Rijn M, Goldberg AB, *et al.* Allergen-induced bronchial hyperreactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. *Proc Natl Acad Sci U S A* 1997; 94: 1344–1349.
25 Nogami M, Suko M, Okudaira H, *et al.* Experimental pulmonary eosinophilia in mice by *Ascaris suum* extract. *Am Rev Respir Dis* 1990; 141: 1289–1295.
26 Takeda K, Hamelmann E, Joetham A, *et al.* Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *J Exp Med* 1997; 186: 449–454.
27 Kraneveld AD, van der Kleij HP, Kool M, *et al.* Key role for mast cells in nonatopic asthma. *J Immunol* 2002; 169: 2044–2053.
28 Kung TT, Stelts D, Zurcher JA, *et al.* Mast cells modulate allergic pulmonary eosinophilia in mice. *Am J Respir Cell Mol Biol* 1995; 12: 404–409.
29 Ogawa K, Kaminuma O, Kikkawa H, *et al.* Primary role of CD4+ T cells and supplemental role of mast cells in allergic pulmonary eosinophilia. *Int Arch Allergy Immunol* 1999; 120: Suppl. 1, 15–18.
30 Grimbaldeston MA, Chen CC, Piliponsky AM, Tsai M, Tam SY, Galli SJ. Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology *in vivo*. *Am J Pathol* 2005; 167: 835–848.
31 Stassen M, Hültner L, Schmitt E. Classical and alternative pathways of mast cell activation. *Crit Rev Immunol* 2002; 22: 115–140.
32 Kinet JP. The high-affinity IgE receptor (FcεRI): from physiology to pathology. *Annu Rev Immunol* 1999; 17: 931–972.

33 Kawakami T, Galli SJ. Regulation of mast-cell and basophil function and survival by IgE. *Nat Rev Immunol* 2002; 2: 773–786.
34 Grünig G, Warnock M, Wakil AE, *et al.* Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998; 282: 2261–2263.
35 Wills-Karp M, Luyimbazi J, Xu X, *et al.* Interleukin-13: central mediator of allergic asthma. *Science* 1998; 282: 2258–2261.
36 Taube C, Duez C, Cui ZH, *et al.* The role of IL-13 in established allergic airway disease. *J Immunol* 2002; 169: 6482–6489.
37 Benyon RC, Bissonnette EY, Befus AD. Tumor necrosis factor-α dependent cytotoxicity of human skin mast cells is enhanced by anti-IgE antibodies. *J Immunol* 1991; 147: 2253–2258.
38 Walsh LJ, Trinchieri G, Waldorf HA, Whitaker D, Murphy GF. Human dermal mast cells contain and release tumor necrosis factor-α, which induces endothelial leukocyte adhesion molecule 1. *Proc Natl Acad Sci USA* 1991; 88: 4220–4224.
39 Zhang Y, Ramos BF, Jakschik BA. Neutrophil recruitment by tumor necrosis factor from mast cells in immune complex peritonitis. *Science* 1992; 258: 1957–1959.
40 Echtenacher B, Männel DN, Hültner L. Critical protective role of mast cells in a model of acute septic peritonitis. *Nature* 1996; 381: 75–77.
41 von Stebut E, Metz M, Milon G, Knop J, Maurer M. Early macrophage influx to sites of cutaneous granuloma formation is dependent on MIP-1α/β released from neutrophils recruited by mast cell-derived TNFα. *Blood* 2003; 101: 210–215.
42 Rijnierse A, Koster AS, Nijkamp FP, Kraneveld AD. TNF-α is crucial for the development of mast cell-dependent colitis in mice. *Am J Physiol Gastrointest Liver Physiol* 2006; 291: G969–G976.
43 Biedermann T, Kneilling M, Mailhammer R, *et al.* Mast cells control neutrophil recruitment during T cell-mediated delayed-type hypersensitivity reactions through tumor necrosis factor and macrophage inflammatory protein 2. *J Exp Med* 2000; 192: 1441–1452.
44 van Houwelingen AH, Kool M, de Jager SC, *et al.* Mast cell-derived TNF-α primes sensory nerve endings in a pulmonary hypersensitivity reaction. *J Immunol* 2002; 168: 5297–5302.
45 Casale TB, Costa JJ, Galli SJ. TNF-α is important in human lung allergic reactions. *Am J Respir Cell Mol Biol* 1996; 15: 35–44.
46 Busse PJ, Zhang TF, Srivastava K, *et al.* Chronic exposure to TNF-α increases airway mucus gene expression *in vivo*. *J Allergy Clin Immunol* 2005; 116: 1256–1263.
47 Choi IW, Sun K, Kim YS, *et al.* TNF-α induces the late-phase airway hyperresponsiveness and airway inflammation through cytosolic phospholipase A(2) activation. *J Allergy Clin Immunol* 2005; 116: 537–543.
48 Nakae S, Lunderius C, Ho LH, Schäfer B, Tsai M, Galli SJ. TNF can contribute to multiple features of ovalbumin-induced allergic inflammation of the airways in mice. *J Allergy Clin Immunol* 2007; 119: 680–686.
49 Kim YS, Ko HM, Kang NI, *et al.* Mast cells play a key role in the development of late airway hyperresponsiveness through TNF-α in a murine model of asthma. *Eur J Immunol* 2007; 37: 1107–1115.
50 Nakae S, Ho LH, Yu M, *et al.* Mast cell-derived TNF contributes to airway hyperreactivity, inflammation, and TH2 cytokine production in an asthma model in mice. *J Allergy Clin Immunol* 2007; 120: 48–55.
51 Erin EM, Leaker BR, Nicholson GC, *et al.* The effects of a monoclonal antibody directed against tumor necrosis factor-α in asthma. *Am J Respir Crit Care Med* 2006; 174: 753–762.
52 Berry MA, Hargadon B, Shelley M, *et al.* Evidence of a role of tumor necrosis factor-α in refractory asthma. *N Engl J Med* 2006; 354: 697–708.
53 Howarth PH, Babu KS, Arshad HS, *et al.* Tumour necrosis factor (TNF-α) as a novel therapeutic target in symptomatic corticosteroid dependent asthma. *Thorax* 2005; 60: 1012–1018.
54 van Rijt LS, Jung S, Kleinjan A, *et al. In vivo* depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J Exp Med* 2005; 201: 981–991.
55 Jawdat DM, Albert EJ, Rowden G, Haidl ID, Marshall JS. IgE-mediated mast cell activation induces Langerhans cell migration *in vivo*. *J Immunol* 2004; 173: 5275–5282.