

Original Paper

Phenotypic Characteristics of Nasal Mast Cells in a Mouse Model of Allergic Rhinitis

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Key Words

Allergic rhinitis · Mast cells · Mice · Serine proteases · IgE · Respiratory mucosa · Olfactory mucosa

Abstract

Background: Mast cells (MCs) in the nasal respiratory mucosa (NRM) play a triggering role in the pathogenesis of allergic rhinitis (AR). Recent research evidence in mouse models of AR suggests an underlying MC-related allergic response in mouse nasal olfactory mucosa (NOM). **Objective:** We sought to investigate the phenotypic characteristics of nasal MCs in a mouse model of AR. **Methods:** By MC-specific staining and immunohistochemistry, we analyzed the subset, protease and IgE-binding phenotypes of nasal MCs in ovalbumin (OVA)-sensitized unchallenged and challenged mice. **Results:** In OVA-sensitized challenged mice, increased serum OVA-specific IgE levels ($p < 0.001$) and eosinophil infiltration confirmed AR induction. In addition to constitutive connective tissue MCs, mucosal MCs were induced in NRM and NOM of OVA-sensitized challenged mice. Connective tissue MCs and mucosal MCs in mouse NRM and NOM were positive for mouse MC protease-1, -4, -5, -6, -7 and carboxypeptidase-A3. In line with MCs in NRM, there were increased numbers ($p = 0.019$) and proportions ($p = 0.027$) of MCs with surface-bound IgE in NOM of OVA-sensitized challenged mice. **Conclusion:** In the setting of AR, MCs in mouse NOM exhibit the same subset, protease and IgE-binding phenotypes as MCs in mouse NRM.

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Introduction

Allergic rhinitis (AR) is characterized by IgE-driven inflammation in the nasal respiratory mucosa (NRM) where mast cells (MCs) and eosinophils are prominent [1, 2]. In the pathogenesis of AR, MCs play a triggering role by acquiring IgE on their surface. MCs are activated

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by the allergen-induced crosslinking of surface-bound IgE, leading to an allergic response in NRM. An abundance of proinflammatory mediators is released from MCs in the early-phase response and promotes eosinophil infiltration into NRM in the later-phase response [3].

In recent years, a few studies have reported that eosinophils infiltrate into the nasal olfactory mucosa (NOM) of AR patients and in mouse models, suggesting a direct and deleterious effect of AR on human and mouse NOM [4–7]. In addition to eosinophils, MCs and plasma cells have been observed in NOM of mouse models, giving rise to the hypothesis of allergic response in mouse NOM [5]. Based on this hypothesis, an important question that remains unanswered is whether these MCs in mouse NOM exhibit the IgE-binding phenotype as a basis for allergic response.

Mouse MCs are usually characterized by their expression of specific proteases, such as the two chymases mouse MC protease (mMCP)-1 and mMCP-4, elastase mMCP-5, the two tryptases mMCP-6 and mMCP-7, together with carboxypeptidase-A3 (CPA3) [8]. Early immunohistochemical studies of the intestinal mucosa of mice with *Trichinella spiralis* infection using antibodies against these proteases have provided the first evidence for the existence of two MC subsets with different locations and expressing proteases: constitutive connective tissue MCs (CTMCs) expressing mMCP-4, -5, -6, -7 and CPA3 and induced mucosal MCs (MMCs) expressing mMCP-1 [9, 10]. Recently, this notion has been reacquainted by the findings that the protease phenotypes of mouse MC subsets vary among tissues [11]. In the airways of ovalbumin (OVA)-sensitized mice undergoing repetitive aerosol OVA challenge, both constitutive CTMCs and induced MMCs in the trachea express mMCP-1, -4, -5, -6, -7 and CPA3. In the proximal bronchi, constitutive CTMCs still express all six proteases, whereas induced MMCs only express mMCP-1, -6 and -7 [8]. To date, the subset and protease phenotypes of mouse nasal MCs have not been defined, especially in the setting of AR.

The heterogeneity of protease phenotypes is attributed to the unique development of mouse MCs in vivo. Bone marrow-derived Kit⁺ (CD117⁺) MC progenitors do not mature until after their recruitment into peripheral tissues, resulting in the specific tissue-regulation of MC protease expression [8]. However, mouse MCs are distinguished from most other myeloid lineage cells by keeping their Kit expression with maturation [12]. Therefore, Kit has been used as an alternative marker for the identification of mouse MCs [13, 14].

In this study, we established a mouse model of OVA-specific AR in an attempt to investigate the phenotypic characteristics of MCs in mouse nasal mucosa. By MC-specific staining and immunohistochemistry, we sought to analyze the subset, protease and IgE-binding phenotypes of MCs in mouse NRM and NOM.

Materials and Methods

Animals

Female BALB/c mice aged 8 weeks (Vital River, Beijing, China) were housed in specific pathogen-free conditions at the animal facility of the Xuanwu Hospital, Capital Medical University. The study protocol conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures in this animal experiment were approved by the Capital Medical University's Institutional Animal Care and Use Committee.

Sensitization and Challenge with OVA

Mice were divided into two groups and each group consisted of 12 mice (OVA-sensitized challenged mice: treatment group; OVA-sensitized unchallenged mice: control group). The mice from both groups were sensitized by an intraperitoneal injection of 10 µg OVA (A-5503, Grade V; Sigma-Aldrich, Shanghai, China) emulsified to 2 mg alum (Sigma-Aldrich) as adjuvant in 0.5 ml phosphate-buffered saline (PBS) on day 0 and day 7. From day 14, OVA-sensitized challenged mice were challenged by 1% aerosol (10 mg/ml in PBS) OVA for 30 min daily for 14 consecutive days. Aerosol OVA challenge simulates the natural route of allergen entry into mouse NOM. However, OVA-sensitized unchallenged mice did not receive aerosol OVA challenge. All mice were sacrificed for sample collection on day 30.

Table 1. Primary antibodies used for immunostaining

Antibody	Host	Dilution	Clone	Catalog No.	Manufacturer
Anti-mMCP-1	Rat	1:200	RF6.1	14-5503	eBioscience, San Diego, Calif., USA
Anti-mMCP-4	Goat	1:200	Polyclonal	ab92368	Abcam, Cambridge, UK
Anti-mMCP-5	Rabbit	1:800	Polyclonal	–	Generous gift from Prof. Michael F. Gurish, Brigham and Women's Hospital, Harvard Medical School, Boston, Mass., USA
Anti-mMCP-6	Goat	1:300	Polyclonal	sc-32474	Santa Cruz Biotechnology, Santa Cruz, Calif., USA
Anti-mMCP-7	Rabbit	1:1,000	EPR8476	ab134932	Abcam
Anti-CPA3	Goat	1:400	Polyclonal	sc-49445	Santa Cruz Biotechnology
Anti-IgE	Goat	1:100	Polyclonal	NB7530	Novus Biologicals, Littleton, Colo., USA
Anti-Kit	Rabbit	1:400	Polyclonal	sc-5535	Santa Cruz Biotechnology

ELISA for Serum OVA-Specific IgE

Mice were deeply anesthetized with 10% chloral hydrate. The cardiac blood was collected by puncture, centrifuged (14,000 rpm, 5 min) and the separated serum was frozen at -80°C . The serum OVA-specific IgE level was measured using a commercially available ELISA kit (#3010; Chondrex Inc., Redmond, Wash., USA) in accordance with the manufacturer's protocols. Briefly, a 96-well plate was coated with anti-IgE (clone 77-1) as capture antibody. After adding mouse IgE standards, blank and serum samples in duplicate, biotinylated OVA was added and the reaction was amplified with streptavidin peroxidase. The optical density of each well was determined using a microplate reader (Model 680; Bio-Rad Laboratories, Hercules, Calif., USA) at a wavelength of 450 nm. A standard curve was created of the average of the duplicate optical density readings. OVA-specific IgE levels were calculated using Excel 2003 (Microsoft).

Tissue Preparation

Anesthetized mice were transcardially perfused with PBS followed by 4% paraformaldehyde solution in PBS. Thereafter, the heads were trimmed, decalcified in 30% formic acid for 14 days and embedded in paraffin after dehydration. According to the murine nasal diagrams by Mery et al. [15], slightly forward-oblique coronal incisions were made through the incisive papilla, second palatal ridge and second upper molar teeth to obtain three nasal cross-sections separated by at least 1 mm. From each nasal cross-section, 4- μm -thick sections were harvested and collected on glass slides for the following procedures.

Histology

After having been rinsed in xylene and ethanol for the removal of paraffin, sections were stained with Mayer's hematoxylin-eosin stain to detect eosinophils and with toluidine blue stain to detect MCs.

Immunohistochemistry

All primary antibodies are listed in table 1. In each staining experiment, sections incubated without antibody were included as negative controls. There was no obvious labeling corresponding to immunostaining by the primary antibodies (data not shown).

Immunohistochemical Staining of MC Proteases

The sections were rehydrated and washed in PBS 3 times for 3 min each. To unmask the antigen, the sections were immersed into 1 mM EDTA (pH 9.0), autoclaved for 2 min and then left at room temperature for cooling. They were incubated with 3% H_2O_2 for 30 min at room temperature to block endogenous peroxidases and then washed. Antibodies against mMCP-1, -4, -5, -6, -7 and CPA3 were incubated overnight at 4°C . For visualization of the staining, an EliVision plus kit (Maixin Bio, Fuzhou, China) was used according to the manufacturer's instructions. The sections were incubated with the postblocking reagent for 20 min at room temperature. After washing, the secondary antibodies of the EliVision detection system were applied for 30 min at room temperature and the sections were washed again. The reaction products were visualized with 3-amino-9-ethylcarbazole (Maixin Bio). Finally, the sections were counterstained with hematoxylin and then mounted using aqueous mounting medium (Maixin Bio).

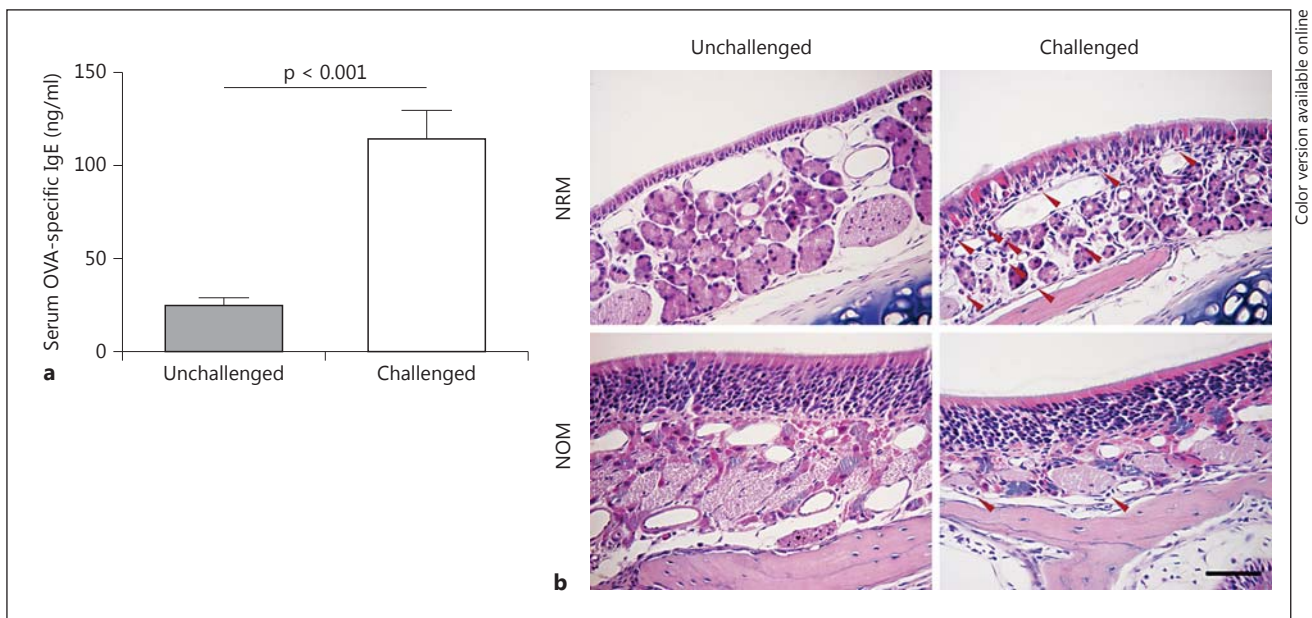


Fig. 1. Establishment of a mouse model of OVA-induced AR. **a** Serum OVA-specific IgE levels from OVA-sensitized unchallenged and challenged mice ($n = 6$ per group). **b** Representative hematoxylin-eosin-stained nasal sections from OVA-sensitized unchallenged and challenged mice. Arrowheads show eosinophils. Scale bar = 50 μm .

Immunohistochemical Identification of IgE⁺ MCs

After antigen retrieval in a pressure cooker, a biotin block kit (Maixin Bio) was used to block tissue endogenous biotin according to the manufacturer's instructions. Kit expression was visualized with a rabbit polyclonal antibody against Kit and a donkey anti-rabbit IgG polyclonal antibody conjugated to Alexa Fluor 488 (A-21206; Molecular Probes, Eugene, Oreg., USA). IgE expression was visualized with a goat polyclonal antibody against IgE, together with a chicken anti-goat biotinylated polyclonal antibody (Abcam, Cambridge, UK) and Alexa Fluor 555-conjugated streptavidin (S-32355; Molecular Probes). Cell nuclei were stained with DAPI (D1306; Molecular Probes).

Quantitative Analysis

From each mouse, six serial sections from each nasal cross-section were examined to quantify nasal MCs. MCs within 200 μm of mouse NRM/NOM transition were excluded. The average number of MCs in each nasal cross-section was added for each mouse. All counting procedures were performed by an independent observer (G.S.) who was blinded to the experimental groups.

Statistical Analysis

All data are shown as means $\pm$ SEMs. Between-group comparisons were made using Student's test with GraphPad Prism software (version 5; GraphPad, Inc., San Diego, Calif., USA). A p value < 0.05 was considered statistically significant.

Results

Establishment of OVA-Induced AR

As two key indicators of AR induction, increased serum OVA-specific IgE levels and prominent eosinophil infiltration into NRM were observed in OVA-sensitized challenged mice but not in OVA-sensitized unchallenged mice (fig. 1).

Serum OVA-Specific IgE

The serum OVA-specific IgE level was low in OVA-sensitized unchallenged mice and significantly increased in OVA-sensitized challenged mice (24.50 ± 4.49 vs. 114.28 ± 15.28 ng/ml; $p < 0.001$; fig. 1a).

Eosinophil Infiltration into Mouse NRM and NOM

By hematoxylin-eosin staining, eosinophils were detected in neither NRM nor NOM of OVA-sensitized unchallenged mice. In OVA-sensitized challenged mice, however, there was a large number of subepithelial eosinophils in NRM and much fewer ones in NOM (fig. 1b).

Subset Phenotypes of MCs in Mouse NRM and NOM

NRM

In OVA-sensitized unchallenged mice, all toluidine blue-positive MCs in NRM were located in the lamina propria. These MCs (CTMCs) were widely scattered around subepithelial vessels and did not exhibit visible degranulation. In contrast, numerous degranulating CTMCs were evident in NRM of OVA-sensitized challenged mice. Together with CTMCs, a few intraepithelial MCs (MMC) without degranulation were observed in NRM of OVA-sensitized challenged mice (fig. 2a). The number of MMCs in NRM of OVA-sensitized challenged mice was 2.00 ± 0.73 . The number of CTMCs in NRM of OVA-sensitized challenged mice was slightly but not significantly higher than that of OVA-sensitized unchallenged mice (27.17 ± 0.75 vs. 30.00 ± 1.11 ; $p = 0.067$; fig. 2b).

NOM

In OVA-sensitized unchallenged mice, all toluidine blue-positive MCs in NOM were located in the lamina propria. These MCs (CTMCs) were sparsely distributed at the bottom of the lamina propria and did not show obvious degranulation. In OVA-sensitized challenged mice, however, a few degranulating CTMCs were found in NOM. Along with CTMCs, intraepithelial MCs (MMC) without degranulation were occasionally observed at the basal cell layer of the olfactory epithelium of OVA-sensitized challenged mice (fig. 2a). The number of MMCs in NOM of OVA-sensitized challenged mice was 1.50 ± 0.47 . The number of CTMCs in NOM of OVA-sensitized challenged mice was slightly but not significantly higher than that of OVA-sensitized unchallenged mice (8.67 ± 0.56 vs. 10.67 ± 0.80 ; $p = 0.068$; fig. 2c).

Protease Phenotypes of MCs in Mouse NRM and NOM

NRM

CTMCs in NRM of OVA-sensitized unchallenged and challenged mice were positive for mMCP-1, -4, -5, -6, -7 and CPA3. These positive CTMCs were in close proximity to subepithelial vessels. The absence of intraepithelial MCs in NRM of OVA-sensitized unchallenged mice was confirmed by immunohistochemistry, whereas MMCs in NRM of OVA-sensitized challenged mice were positive for all six proteases (fig. 3; left panel).

NOM

CTMCs in NOM of OVA-sensitized unchallenged and challenged mice were positive for mMCP-1, -4, -5, -6, -7 and CPA3. These positive CTMCs were primarily located at the bottom of the lamina propria. In contrast to the lack of positive intraepithelial MCs in NOM of OVA-sensitized unchallenged mice, MMCs in NOM of OVA-sensitized challenged mice were positive for all six proteases (fig. 3; right panel).

IgE-Binding Phenotypes of MCs in Mouse NRM and NOM

Both Kit and IgE immunoreactivity displayed a characteristic membrane staining in the double-stained immunofluorescence sections (fig. 4a).

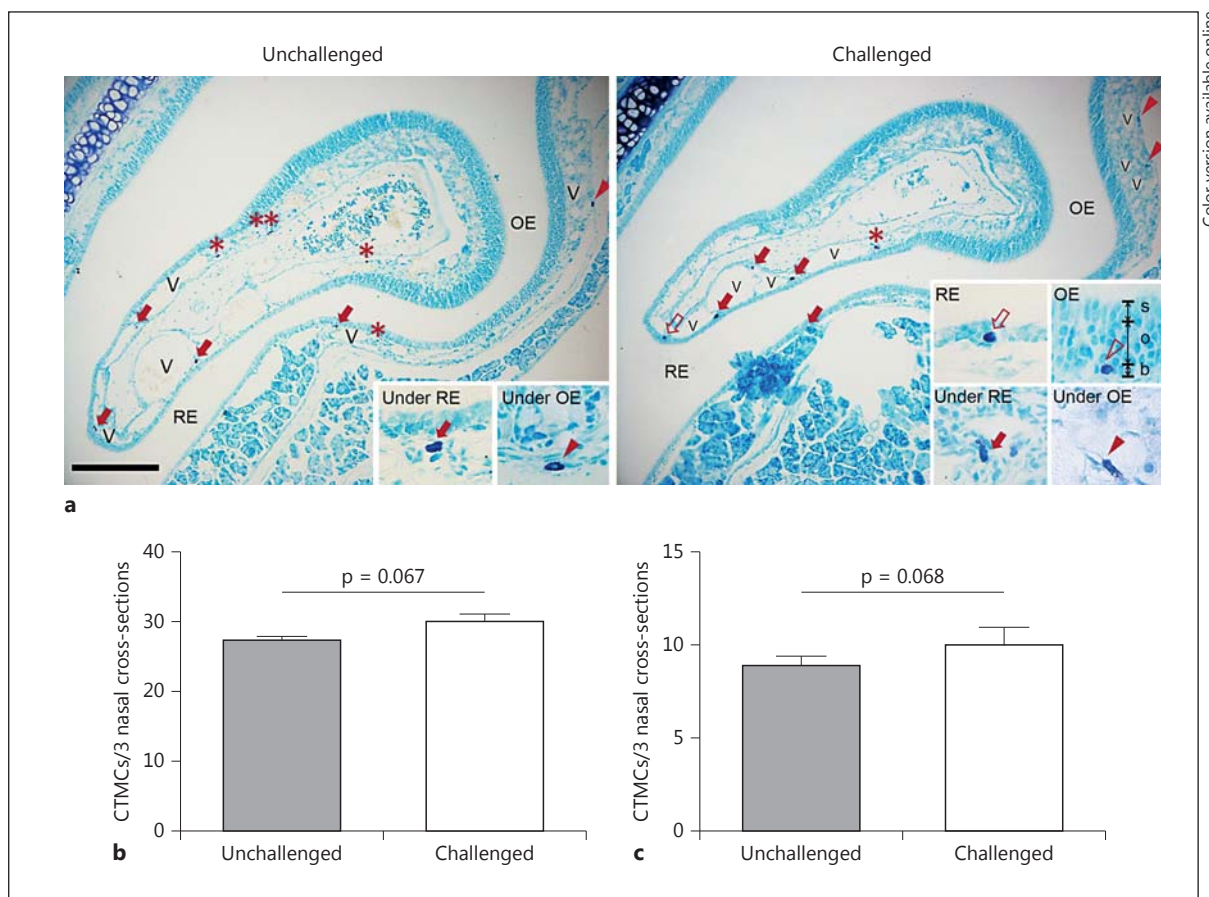


Fig. 2. Subset phenotypes of MCs in mouse NRM and NOM. **a** Toluidine blue-positive MCs in representative nasal sections from OVA-sensitized unchallenged and challenged mice. **Insets** represent images of MC subsets in NRM and NOM from OVA-sensitized unchallenged and challenged mice ($\times 400$). Arrows and empty arrows show CTMCs and MMCs in mouse NRM, respectively; arrowheads and empty arrowheads show CTMCs and MMCs in mouse NOM, respectively; asterisks show CTMCs in mouse NRM/NOM transition. OE = Olfactory epithelium; RE = respiratory epithelium; b = basal cell layer; o = olfactory sensory neuron layer; s = supporting cell layer; v = subepithelial vessels. Scale bar = 200 μ m. **b** CTMCs in NRM from OVA-sensitized unchallenged and challenged mice ($n = 10$ per group). **c** CTMCs in NOM from OVA-sensitized unchallenged and challenged mice ($n = 10$ per group).

NRM

The number of IgE⁺ MCs in NRM was low in OVA-sensitized unchallenged mice and significantly increased in OVA-sensitized challenged mice (4.43 ± 1.51 vs. 20.14 ± 3.67 ; $p < 0.001$). The proportion of IgE⁺ MCs in NRM was also significantly increased in OVA-sensitized challenged mice compared with OVA-sensitized unchallenged mice (17.74 ± 2.44 vs. $71.13 \pm 10.05\%$; $p < 0.001$; fig. 4b).

NOM

The number of IgE⁺ MCs in NOM was low in OVA-sensitized unchallenged mice and significantly increased in OVA-sensitized challenged mice (2.00 ± 1.41 vs. 4.14 ± 1.68 ; $p = 0.019$). Also, an increase in the proportion of IgE⁺ MCs in NOM was found in OVA-sensitized challenged mice compared with OVA-sensitized unchallenged mice (16.18 ± 10.80 vs. $29.31 \pm 9.42\%$; $p = 0.027$; fig. 4c).

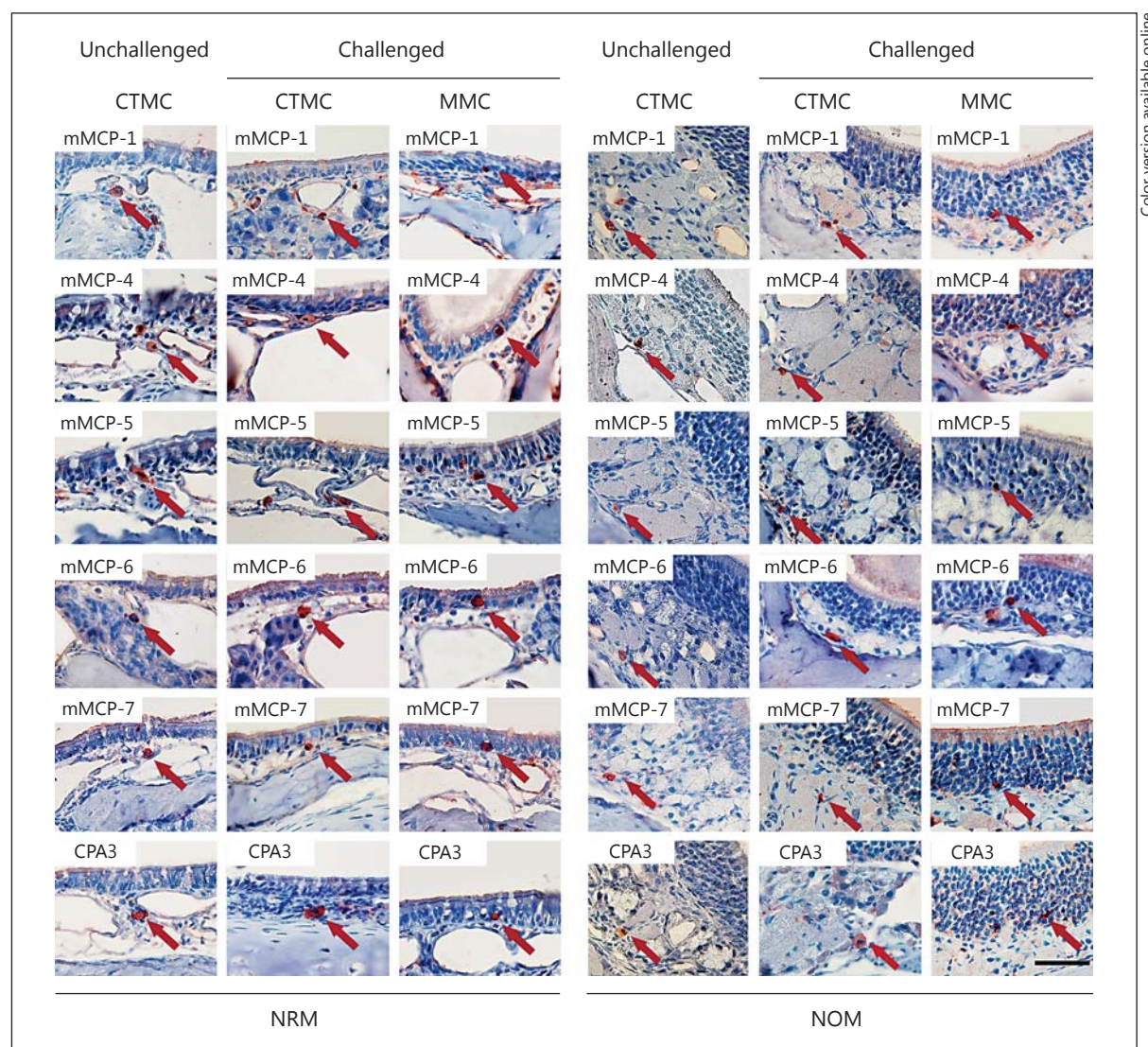


Fig. 3. Protease phenotypes of MCs in mouse NRM and NOM. Representative images from the analysis of 6 mice per group from three separate experiments are shown. Arrows show positive MCs. Scale bar = 50 μ m.

Discussion

In this study, we established a mouse model of OVA-specific AR to identify MCs in NRM and NOM. Taken together, we demonstrated for the first time that (1) in addition to constitutive CTMCs, intraepithelial MMCs were induced in mouse NRM and NOM under AR conditions; (2) CTMCs and MMCs in mouse NRM and NOM expressed the same proteases of mMCP-1, -4, -5, -6, -7 and CPA3, and (3) in the setting of AR, MCs with surface-bound IgE were increased in mouse NRM and NOM.

As a constitutive subset of mouse MCs, CTMCs are resident in peripheral tissues and responsible for pathogen surveillance [8, 16]. In this study, we found CTMCs in the lamina propria of NRM and NOM of OVA-sensitized unchallenged mice. Considering the hematopoietic origin of mouse MCs, this finding indicates that a number of bone marrow-derived

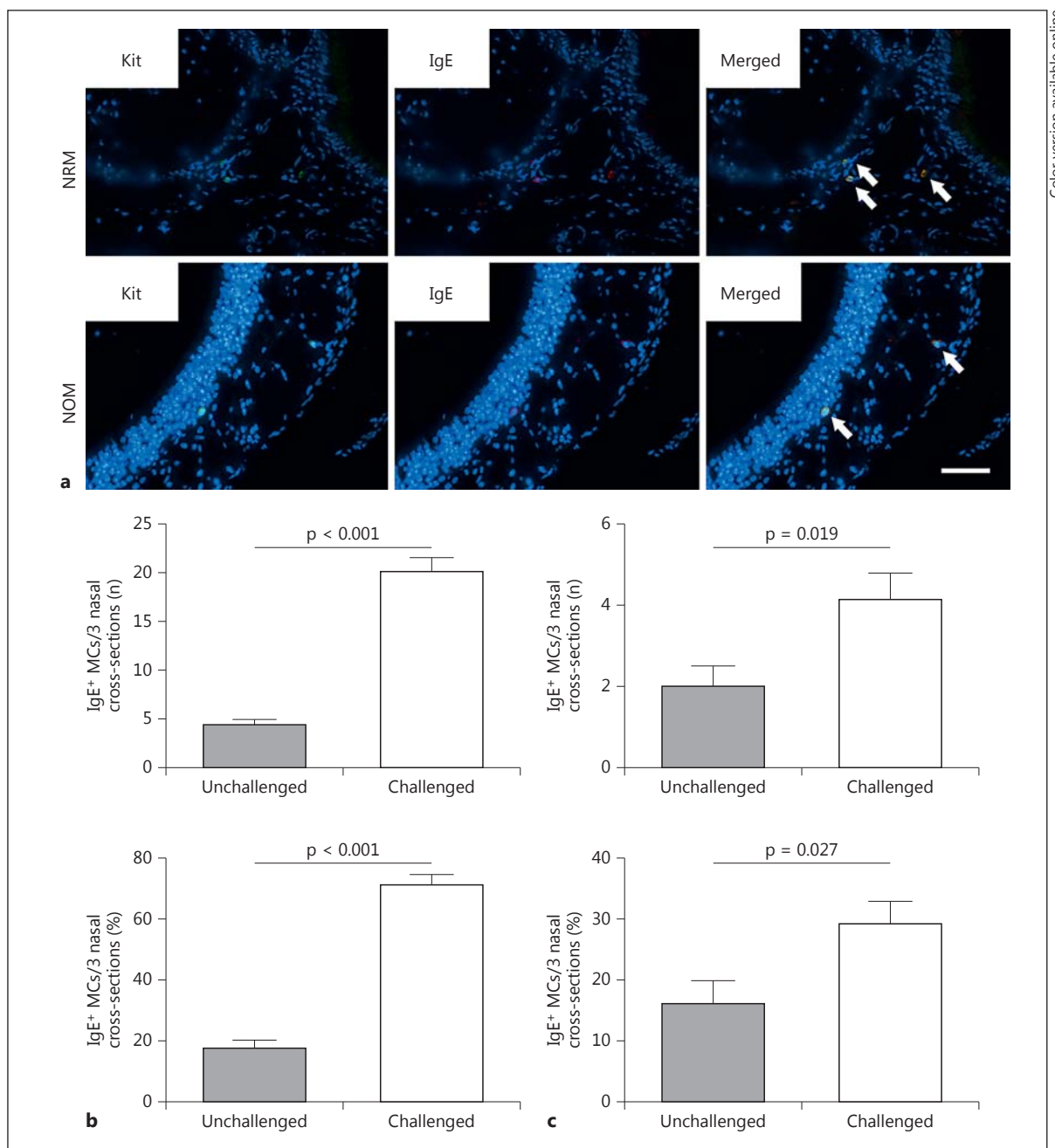


Fig. 4. IgE-binding phenotype of MCs in mouse NRM and NOM. **a** Representative immunofluorescence of IgE⁺ MCs in NRM and NOM from OVA-sensitized challenged mice. Arrows show IgE⁺ MCs. Scale bar = 50 μ m. The number and proportion of IgE⁺ MCs in NRM (**b**) and NOM (**c**) from OVA-sensitized unchallenged and challenged mice (n = 7 per group).

MC progenitors are recruited into mouse NRM and NOM. Moreover, we noticed that most CTMCs in mouse NOM were located at the bottom of the lamina propria rather than scattered around the subepithelial vessels as CTMCs in mouse NRM. This difference implies that the mechanism mediating the localization of CTMCs in mouse NOM is distinct from that in mouse NRM.

The number of CTMCs in NRM and NOM from OVA-sensitized challenged mice was slightly but not significantly increased compared to that from OVA-sensitized unchallenged mice. In addition to CTMCs, intraepithelial MMCs were induced in mouse NRM and NOM. To date, it still remains uncertain whether the appearance of MMCs results from the differentiation of MC progenitors that are newly recruited into tissues or from the redistribution of existing CTMCs that migrate into the epithelium. However, the induction of MMCs is considered to be T cell dependent, because of their absence in the intestinal mucosa of athymic mice with *Trichinella spiralis* infection [17]. Therefore, a Th2-skewed allergic response in OVA-sensitized challenged mice might drive the appearance of MMCs in NRM and NOM.

Recently, it has been recognized that the protease phenotypes of mouse MCs are regulated by local tissues [11]. In this study, we demonstrated that CTMCs and MMCs in mouse NRM and NOM express the same proteases of mMCP-1, -4, -5, -6, -7 and CPA3. In vitro studies of mouse bone marrow-derived MCs have revealed that the protease expression of mouse MCs is cytokine dependent. For example, TGF- β 1 mediates the expression of mMCP-1, whereas IL-33 promotes the expression of mMCP-6 in mouse MCs [18, 19]. Based on these results, it has been deduced that the specific tissue regulation of protease phenotypes of mouse MCs is accomplished through their surrounding cytokine milieu in the tissue microenvironment [8]. In mouse NRM, TGF- β 1 and IL-33 are produced under normal conditions and upregulated in the setting of AR [20, 21]. These findings might account for the expression of mMCP-1 and -6 in constitutive CTMCs and induced MMCs in mouse NRM. Our findings also indicate that similar cytokines might be involved in the regulation of protease expression of MCs in mouse NOM.

MCs in NRM play a triggering role in the pathophysiology of AR by their acquisition of IgE on the cell surface [3]. Consistent with this notion, the number and proportion of IgE⁺ MCs in NRM were increased in OVA-sensitized challenged mice compared with OVA-sensitized unchallenged mice. Interestingly, we also detected increased IgE⁺ MCs in NOM of OVA-sensitized challenged mice. MC-bound IgE in mouse NOM might derive from blood-borne IgE through passive diffusion across the vasculature. On the other hand, there might be a local production of IgE by infiltrated plasma cells in mouse NOM [5]. In vivo and in vitro studies have demonstrated that surface-bound IgE is able to reduce the apoptosis of MCs irrespective of antigen [22, 23]. To a certain extent, the IgE-binding phenotype of MCs contributes to the increase in numbers and proportions of MCs in NOM and NRM of OVA-sensitized challenged mice.

Importantly, the activation of MCs with surface-bound IgE depends on the allergen-induced crosslinking of IgE. Therefore, we did not see degranulating MCs in NRM or NOM of OVA-sensitized unchallenged mice despite a number and proportion of MCs showing IgE-binding phenotype. In contrast, CTMCs undergoing degranulation were observed in NRM and NOM of OVA-sensitized challenged mice 2 days after the last OVA challenge. This finding demonstrates the activation-inducing effect of local antigen challenge on nasal MCs. The detection of degranulating CTMCs in NOM of OVA-sensitized challenged mice suggests that MC-bound IgE in mouse NOM might be able to recognize deposited allergen, although most CTMCs in mouse NOM were located at the bottom of the lamina propria. If so, this is technically difficult to demonstrate because of the rapid endocytosis of allergen by MCs [24]. On the one hand, the activation of CTMCs in NOM of OVA-sensitized challenged mice probably leads to the release of proinflammatory mediators into subepithelial vessels and the recruitment of eosinophils into mouse NOM as we have observed. On the other hand, degranulating CTMCs in NOM of OVA-sensitized challenged mice imply an underlying detrimental impact on olfactory sensory neurons. In the guinea-pig ileum, MC tryptase has been shown to activate submucosal neurons and to induce local neuroinflammation by cleaving protease-activated receptor 2 expressed on neurons [25]. In addition, it has been demonstrated that protease-

activated receptor 2 is expressed by rat olfactory sensory neurons [26]. However, whether mouse olfactory sensory neurons could be affected by the tryptases mMCP-6 and -7 in CTMCs needs to be further explored.

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