

Conditional depletion of mast cells has no impact on the severity of experimental epidermolysis bullosa acquisita

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Abbreviations

BP, bullous pemphigoid; COL7, type VII collagen; CTMC(s), connective tissue mast cell(s); DT, diphtheria toxin; EBA, epidermolysis bullosa acquisita; i.d., intradermal(ly); MC, mast cell(s); Mcpt5, mast cell protease 5; W-sh, C57BL/6J-*Kit*^{W-sh/W-sh}; W/W-v, WBB6F₁-*Kit*^{W/W-v}

Abstract

The role of mast cells (MCs) in autoimmunity is the matter of an intensive scientific debate. Based on observations in different MC-deficient mouse strains, MCs are considered as fundamental players in autoimmune diseases. However, most recent data suggest that the outcome of such diseases is strongly affected by the individual mouse strain used. By the use of two *c-Kit* mutant mast cell-deficient mouse strains and one *c-Kit*-independent strain, we here investigated the role of MCs in a systemic antibody transfer model of epidermolysis bullosa acquisita (EBA), a subepidermal autoimmune blistering skin disease characterized by autoantibodies against type VII collagen. While C57BL/6J-*Kit*^{W-sh/W-sh} mice developed an unexpected increased blistering phenotype, no significant differences to wildtype controls were seen in WBB6F₁-*Kit*^{W/W-v} or the novel *Mcpt5-Cre iDTR* animals. Interestingly, in a local

antibody transfer model, which induces a localized disease, we showed that application of high concentrations of anti-COL7 antibodies induced MC activation and MC-dependent edema formation which did, however, not contribute to blister induction. Our results indicate that in the autoimmune disorder EBA MCs do not contribute to the immune-mediated tissue injury. Modern *c-Kit* mutant-independent MC-deficient mouse strains will help to further redefine the role of MCs in autoimmunity.

Introduction

Autoimmune bullous dermatoses represent a group of organ-specific autoimmune diseases with autoantibodies targeting desmosomal and hemidesmosomal structural proteins of skin and mucous membranes. Within this group, epidermolysis bullosa acquisita (EBA) represents a subepidermal blistering disorder characterized by circulating and tissue-bound autoantibodies (mostly of the IgG subclass) against type VII collagen (COL7), a major component of anchoring fibrils essential for the attachment of epidermis and dermis [1],[2]. The pathogenicity of anti-COL7 antibodies in EBA has been well documented, both in vitro and in vivo[3]. Studies in men and mice revealed that the deposition of anti-COL7 IgG at the dermal-epidermal junction results in complement activation predominantly via the alternative pathway and subsequent neutrophil infiltration into the dermis of the skin. By releasing reactive oxygen species and proteases, activated neutrophils are thought to destroy the basement membrane zone and mediate dermal-epidermal separation and blister formation [4],[5]. Thus, patients with EBA mainly suffer from fragile skin, blistering, and scarring with milia formation[6]. Although therapeutic approaches include systemic corticosteroids, immunosuppressants, colchicine, dapsone, and high-dose intravenous immunoglobulins [4], therapeutic strategies that effectively help the majority of EBA patients are still missing.

The current research on EBA uses, among others, a mouse model where experimental EBA is induced by transfer of pathogenic rabbit anti-murine collagen 7-IgG (anti-COL7) [7],[8] and allows the investigation of innate effector mechanisms, in EBA.

Mast cells (MCs) are one of the predominant innate immune cells in the non-inflamed skin and act as first-line defenders on interfaces of our body. They combat invading pathogens, parasites and harmful antigens by releasing pre-stored histamines and proteases, new synthesized lipid mediators and cytokines. Thus, MCs are well equipped to initiate and maintain inflammatory responses and to recruit leukocytes to the site of infection[9]. Thereby, MCs cannot be exclusively activated by cross-linking of Fc_ϵ receptors, the major trigger in allergy but also by a variety of stimuli like IgG immune complexes or complement factors [10],[11], which are also present during the onset of EBA.

MCs derive from hematopoietic progenitors and complete their maturation only in the target tissues under the influence of the stem cell factor. The binding of stem cell factor by its receptor c-Kit is essential for the development and survival of MCs[12]. Based on mutations in the *c-Kit* gene, different mast cell-deficient (MC-deficient) mouse strains were bred. Among them, the C57BL/6-*Kit*^{W-sh/W-sh} strain (W-sh) and the WBB6F₁-*Kit*^{W/W-v} strain (W/W-v) are best established in the field of MC research and thus, are the best investigated MC-deficient mice with a constitutive lack of MCs[13],[14]. In addition to these *c-Kit* mutant-mice, conditional MC-deficient strains were developed like the Cre-Master [15] or the double transgenic *Mcpt5-Cre iDTR* mice (Cre/iDTR) [16],[17]. In the latter, the administration of diphtheria toxin results in a specific depletion of connective tissue MC in adult mice.

Using different MC-deficient mouse strains, MCs, previously characterized as central players in allergy, were then recognized as important regulators in autoimmune diseases. Several studies could allocate a disease-promoting function of MCs in experimental models of rheumatoid arthritis, multiple sclerosis and bullous pemphigoid (BP) [18]–[21], making them an attractive target for novel therapeutic strategies. However, newly generated *c-Kit* mutant-independent MC-deficient mice like the transgenic *Mcpt5-Cre iDTR* strain redefined the involvement of MCs in autoimmune diseases. Consequently, in this study, we investigated the role of MCs in a local and a systemic model of experimental EBA using constitutive and conditional MC-deficient strains. Although we demonstrate an activation of MCs in these EBA models, MCs are not a prerequisite for the antibody-induced tissue injury.

Results

Mast cells are degranulated in skin of EBA patients

To get a first hint for the potential involvement of mast cells in the human EBA, we examined the histology of skin samples derived from five different patients suffering from the inflammatory type of the disease. In lesional skin samples of EBA patients stained for NaphtholAS-D chloroacetate esterase, MCs were clearly degranulated while in healthy human skin exclusively resting MCs were observed (Fig. 1). These results provide clear evidence that MCs are activated during EBA and indicate a potential role for these cells in the pathogenesis of the disease.

Mast cell-deficient mice are not protected from experimental EBA

In the next step, we induced experimental EBA by the transfer of pathogenic anti-COL7 IgG [7]. For determining the principal functional role of MCs in experimental EBA, we investigated first two constitutive MC-deficient mouse strains, C57BL/6J-*Kit*^{W-sh/W-sh} (W-sh) and WBB6F₁-*Kit*^{W/W-v} (W/W-v). After repeated administration of pathogenic anti-COL7 IgG, all mice were scored for disease outcome on days 4, 8, 12, and 16. All strains developed a subepidermal blistering disease duplicating the findings in patients with the inflammatory variant of EBA within four days after first antibody injection. Unexpectedly, mice of the W-sh strain developed a more severe phenotype of experimental EBA as compared with the corresponding wildtype controls which became visible already at day four after the first injection and increased further up to day 16 of the experiment (Fig.2A). By contrast, MC-deficient mice of the second constitutive strain, W/W-v, developed a similar clinical disease compared with their littermates over the entire period of the experiment (Fig.2B). It should be noted that W/W-v mice and their littermates presented a milder EBA phenotype than the C57BL/6J and W-sh mice (AUC medians: W/W-v 7.74/littermates 10.91 vs. W-sh 32.64/WT 21.25; $p < 0.0001$, Kruskal-Wallis test), which may be due to the different genetic background of the mice.

Based on these contradictory results, we could not exclude that the increased pathology present in W-sh mice was simply not seen in the W/W-v strain due to side effects of the *c-Kit* mutation. To address this discrepancy, experiments were performed in the recently described double transgenic *Mcpt5-Cre iDTR* (Cre/iDTR) strain. In these mice the connective tissue mast cells (CTMCs) can be ablated by the application of low doses of diphtheria toxin (DT) without causing unwanted side effects on other cell populations[17]. These double transgenic

Cre/iDTR mice were pretreated with DT four times in a weekly interval for the depletion of CTMCs, or with PBS for mast cell-sufficient (MC-sufficient) littermates. DT-treatment alone did not affect the disease activity in Cre negative, iDTR-positive mice (data not shown). By applying this protocol we achieved an average depletion rate of 84% of all dermal MC. However, the percentage of depleted MCs varied in different anatomical sites (ear: 77%; face: 86%; feet: 79%; trunk: 88%, tail: 90%). Between day 12 and day 16 after administration of the pathogenic anti-COL7 IgG, MC-deficient mice developed a slight but not significant milder pathology as compared with MC-sufficient mice (Fig.2C) confirming the findings of the W/W-v strain. Even induction of a more severe EBA phenotype revealed no impact of MC deficiency on disease outcome (day 16: PBS $32.4\% \pm 6$ vs. DT $38.0\% \pm 3$; $p = 0.246$). In summary, our results show that in this systemic passive model the pathogenesis of experimental EBA develops independently of MCs.

Since neutrophils are the main effector cells in EBA [22], the percentage of these cells in whole blood was analyzed before EBA induction in mice. Flow cytometric measurements of blood samples revealed equal ratios of neutrophils in W-sh or Cre/iDTR mice and their respective wildtype controls. However, a reduced percentage of neutrophils was detected in blood of W/W-v mice compared with their littermates (Supplementary Fig. S1A).

EBA pathology was further evaluated by analysis of skin histology at day 16 when skin samples of areas with alopecia, crust and erosion were collected for paraffin- and cryosections. As shown by immunofluorescence staining, the deposition of anti-COL7 IgG and complement component C3 could be detected and both were co-localized at the dermal-epidermal junction zone (Fig.2A). Furthermore, the infiltration of neutrophils into the dermis of the skin, subepidermal blistering, and crust formation was observed in Naphthol AS-D chloroacetate esterase stained sections (Fig.3B). Here, quantification of infiltrated neutrophils

in lesional skin revealed no statistically significant differences in numbers of cells between MC-sufficient and MC-deficient groups (Supplementary Fig. S1B). MCs were only present in skin sections of wildtype mice (C57BL/6J, WBB6F₁-Kit^{+/+}, PBS-treated Cre/iDTR) and not detectable in MC-deficient mice (C57BL/6J-Kit^{Wsh/Wsh}, WBB6F₁-Kit^{W/W^v}) or significantly diminished in DT-treated Cre/iDTR mice. Detectable MCs in MC-sufficient mice were not degranulated (data not shown).

These histological observations were consistent in all three MC-deficient mouse strains and their corresponding wildtype controls indicating that the MC-deficient strains show only quantitative but no qualitative differences in the inflammatory process and outcome of EBA.

Mast cells are activated but dispensable in the local disease model

The role of MCs in autoimmune bullous diseases has primarily been investigated for experimental BP by the use of local passive models, where the pathology is induced by a single intradermal injection of high doses of antibody[19]. To elucidate whether the different roles of MCs in BP and EBA represent marks of two distinct bullous diseases or are due to the mode of pathogenic antibody administration, Cre/iDTR mice were analyzed in a local model of EBA in the ear.

Since the analysis of the following experiments exclusively concentrated on the ears, we wanted to make sure to eliminate all resident CTMC. By applying DT additionally into the ear base we achieved a depletion rate of >99% of total MCs.

In addition to the EBA pathology, the local MC activation was assessed by edema formation determined by extravasation of Evans blue 10 minutes after application of pathogenic IgG in the left and control IgG in the right ear. Irrespective to the specificity of the antibody, dark blue coloring indicated a massive edema formation in MC-sufficient mice which could not be

observed in MC-ablated animals (Supplementary Fig. S2). The mechanism for this unspecific activation is not clear but may be related to the high local concentration of antibodies or simply caused by mechanical stress at the site of injection. More importantly, development of disease did not follow activation of MCs. Comparable to the results obtained in the systemic model, mice which had received pathogenic anti-COL7 IgG, but not control IgG, showed a skin pathology irrespective whether mice contained MCs or not (Fig. 4A, B). Although compared with the MC-sufficient group disease severity in MC-deficient mice was significantly milder at day 1, this effect was no longer visible at day 2 where both groups developed disease to the same extent. Moreover, activation of MCs alone as seen in ears receiving control IgG was not sufficient to induce EBA symptoms. Thus, MCs may slightly accelerate the initial inflammatory processes in EBA but do not essentially affect the final state of the disease. Furthermore, histological examination revealed co-localization of anti-COL7 IgG and complement component C3 at the dermal-epidermal junction zone and a subsequent infiltration of neutrophils into the inflamed skin (Fig. 4C), however, independent of the presence of MCs (data not shown).

Discussion

Several studies in human disease and animal models allocated an essential role of MCs to autoimmunity [21],[23]–[27]. However, with the development of novel MC-deficient mouse strains, the contribution of MCs to this type of disorders became questionable and is now under re-evaluation for individual autoimmune diseases.

By the use of an antibody transfer model, we investigated for the first time the role of MCs in experimental EBA. After local application of antibodies, we observed a massive MC degranulation resulting in an increased vascular permeability and edema formation. However, using three different MC-deficient mouse strains we could show that MCs have no relevant impact on blister formation in experimental EBA although MCs activation could be seen in the human disease. Thereby, we determined contradictory results between the two constitutive MC-deficient strains which were also observed in other models of autoimmune diseases like rheumatoid arthritis (RA) and experimental autoimmune encephalomyelitis (EAE). In antibody-induced RA, W/W-v mice were found to be protected from disease while no effect was observed in W-sh mice [20]. Furthermore, in EAE, W/W-v mice developed a milder disease with later onset [18] while in W-sh mice EAE was more severe than in controls [28],[29]. These discrepancies underline the difficulty to discriminate between those parts of a phenotype related to *c-Kit* mutations and those associated with MC-deficiency. In both strains, the receptor c-Kit is altered by the induction of a mutation either in the *c-Kit* gene leading to the expression of non-functional receptors (W/W-v) [30],[31] or in its promoter region disabling a regular expression of the molecule (W-sh) [32],[33]. However, since c-Kit is not only essential for the development of MCs but also involved in the maturation of various other immune cells, side effects not related to MC deficiency[34] could cause a difference in the outcome of experiments [34],[35]. Thus, conflicting results between both mouse strains may be due to differences in the composition of immune cells between both strains or to the individual immune status within these strains [36]. Indeed, we found a reduced number of neutrophils in W/W-v mice compared with their littermates and thus, confirmed former findings of alterations in the number of blood neutrophils [20],[37].

However, instead of a previously described neutrophilia in W-sh mice we observed comparable levels of neutrophils in W-sh mice and their respective wildtype controls[38].

Consequently, as in RA and EAE, the neutropenia in W/W-v mice or the previously reported neutrophilic, proinflammatory phenotype of W-sh mice [38] may affect the outcome of a neutrophil-dependent disease like the inflammatory, bullous pemphigoid-like form of EBA. Thus, results generated with these two mouse lines have to be carefully revisited and need to be re-evaluated by models, where the loss of MCs does not touch other parts of the immune system. The recently described transgenic *Mcpt5-Cre/iDTR* double positive strain represents such a new tool since, besides an inducible deficiency in CTMC, no further immunological defects are reported[16],[39]. These mice express the simian diphtheria toxin receptor (DTR) selectively on CTMCs and thus, CTMCs can be ablated by the application of low doses of DT in normally developed adult mice without affecting other cell populations [17]. Although full MC depletion can normally not be achieved by this model reduction reaches sufficient high levels to identify MC-dependent processes [17]. Recovery of the CTMC population in the skin takes several weeks and therefore this mouse line is sufficient for acute disease models. Thus, currently, the conditional *Cre/iDTR* strain represents the most reliable model to investigate mast cell function in skin *in vivo*. Like Dudeck *et al.* observed in a mouse model of contact hypersensitivity[17], the *Cre/iDTR* line opposes previous findings in the W/W-v and W-sh strains and a selective dependency on *c-Kit* but not on MCs has recently been shown for autoantibody-induced arthritis, too [15]. Both studies illustrate the alterations of the immune system of these *c-Kit* mutant mice beyond the mast cell deficiency.

It should be mentioned that our experimental models are limited in two aspects. First, we addressed here only the inflammatory subtype of EBA and we are not able to argue about the role of MCs in the context of the non-inflammatory form of the disease. Second, although our passive transfer models represent many aspects associated with the effector phase of the human disorder, they are not suitable to analyze the function of MCs in the initial stages of disease development. Within these limitations we, therefore, conclude that MCs are dispensable for the effector phase of experimental EBA.

Of course, in MC-deficient mouse models and in any other knockout model, too, it is difficult to rule out a role for the targeted component in a disease when no phenotype is seen. Compensatory mechanisms in MC-deficient individuals may reconstitute pathways normally driven by MCs [40], which then cannot be identified by our experimental systems. However, such compensatory mechanisms would also apply in therapy. Based on our data, we could provide no evidence for a role of MCs in this disease model.

The disease promoting role of MCs in autoimmune bullous diseases has nearly exclusively been explored for experimental BP. Here, local antibody transfer models were used, where the locally restricted pathology is induced by a single intradermal injection of high doses of antibody within 24 hours[19],[41], differing from the systemic disease model where successive subcutaneous injections of the antibody result in non-localized blister induction. Both passive disease models allow the examination of innate effector mechanisms. However, the advantage of the local disease model, besides saving antibody and time, is the investigation of initial mechanisms of the disease at defined body regions.

Although the pathogenesis of EBA and BP is closely related owing to the involvement of complement and neutrophils[19],[42], our data generated by the systemic disease model suggest no impact of MCs on EBA development and contrast previous findings published for the local model of BP, where neonatal MC-deficient *Mgf^{Sl/Sl-d}* and adult WBB6F₁-*Kit^{W/W-v}* mice were fully protected from the disease [19]. Thus, by analyzing Cre/iDTR mice in a local model of EBA, we elucidated whether the different roles of MCs in BP and EBA represent marks of two distinct bullous diseases or are due to the mode of pathogenic antibody administration. Since in the local EBA model MCs may only accelerate the initial inflammatory response without affecting the final state of the disease, our data suggest that EBA and BP are two bullous diseases whose pathomechanisms decisively differ in the participation of MCs.

Taken together, by using three different MC-deficient mouse strains and two different models of EBA we show that MCs are activated but dispensable for the pathogenesis of the disease. This suggests that, although both autoantibody-mediated bullous disorders, EBA and BP, are closely related, their pathogenesis may involve different immune cells and molecular mechanisms.

Furthermore, with regard to our discrepant results obtained with W/W-v and W-sh mice, conclusions drawn on findings with MC-deficient mice bearing mutations in *c-Kit* must be evaluated carefully. Experiments with *c-Kit-mutant* animals require controls where MCs are repopulated by adoptive transfers as described by Galli and Kitamura [40]. Yet, an exact reconstitution of MCs is difficult and can in skin only be done locally [43],[44]. But since the precise locations of disease outbreaks are unpredictable in systemic models such strains are limited to investigate locally induced inflammation. Thus, currently, the conditional Cre/iDTR strain may represent the most reliable model to investigate functions of connective tissue mast cells *in vivo*.

Materials& Methods

Human skin

Skin biopsies of EBA patients with inflammatory type of EBA were derived from the national reference Centre for Blistering Diseases of the Netherlands and disease was diagnosed as described earlier (no. 9, 11, 29, 31, 32)[45]. All patients gave written informed consent. Cryosections were stained for Naphthol AS-D chloroacetate esterase according to manufacturer's protocol (Sigma-Aldrich, Steinheim, Germany).

Mice

Mcpt5-Cre mice (C57BL/6 background) crossed to the iDTR line (generates double transgenic Cre/iDTR strain [16],[17]), and C57BL/6J-*Kit*^{W-sh/W-sh} (W-sh) were bred and housed at the animal facilities of the Research Center Borstel under specific pathogen-free conditions while WBB6F₁-*Kit*^{W/W-v} (W/W-v) mice and their littermates were purchased from The Jackson Laboratory (Bar Harbor, ME). Nine- to fourteen-week-old mice were used for experiments and littermates served as controls. All animal studies have been reviewed and approved by the Animal Research Ethics Board of the Ministry of Environment, Kiel, Germany, permission no. V312-72241.123-3 (94-9/08) and V312-72241.123-3 (71-5/12).

Induction of mast cell depletion

Ablation of MCs in six- to nine-week-old Cre/iDTR mice was performed by four peritoneal DT injections as described [17] and DT-treated mice displayed a loss of >80% of MCs in the skin, as determined by histological staining for Naphthol AS-D chloroacetate esterase. In addition, mice in the local EBA model received two further subcutaneous injections in the base of the ear (5 ng DT/g body weight) six and two days before antibody application. Compared with littermates, DT-treated Cre/iDTR mice lost >96% of MC in the ears.

Antibody transfer model of EBA

Rabbit anti-COL7 IgG was raised against recombinant proteins of the NC1 domain of murine COL7 as described [7] and purified by protein G affinity chromatography. Systemic disease was induced as described earlier [7] with minor modifications. Mice were treated s.c. with 25 µg IgG/g body weight in the abdominal region on days 0, 2, 4, 6, 8, and 10 followed by a clinical scoring on days 4, 8, 12, and 16.

For local EBA induction, mice were treated as described earlier [41] with minor modifications. In brief, mice received 0.6 mg anti-COL7 IgG intradermally in the base of the ear and were scored 24 h and 48 h later. At this concentration, the antibody induced after 48 h approximately half of the maximal inducible disease score. Pathogenic anti-COL7 IgG was injected into the left, control IgG into the right ear. Edema formation was analyzed after i.v. injection of 200 µl 1% Evans Blue (in PBS). Disease severity was expressed as percentage of total body surface area (systemic model) or surface area of the ear (local model) covered by skin lesions (blisters, crust, erythema). That is, each body region was assigned its percentage on the total body and this value was multiplied by the portion of lesional skin of this body region. The values of all body regions per mouse were summed up and represent the disease score "% of affected skin".

Blood and tissue material was collected on the last day of the experiment.

Histopathology

Naphthol AS-D chloroacetate esterase staining was performed on paraffin sections according to manufacturer's protocol (Sigma-Aldrich, Steinheim, Germany). Deposition of rabbit IgG and C3 was detected on frozen sections of skin specimen by immunofluorescence staining using polyclonal DyLight649-conjugated donkey anti-rabbit IgG (Dianova, Hamburg, Germany) for detection of rabbit IgG and a rat anti-murine C3 antibody (IgG2a; clone RMC11H9; Biozol, Eching, Germany) followed by staining with an AlexaFluor488-conjugated polyclonal goat anti-rat IgG antiserum (Invitrogen, Darmstadt, Germany) for detection of complement component C3.

Quantification of neutrophils in skin tissue

Naphthol AS-D chloroacetate esterase stained paraffin sections of lesional skin tissue of MC-sufficient and MC-deficient mice were chosen. Neutrophils were counted per high power field in three different fields per section, two sections of lesional skin per mouse, and three mice per experimental group. Estimated neutrophil numbers were calculated as number of neutrophils per square mm.

Statistical analysis

Statistical analysis was performed with Prism software (GraphPad Software Inc.). Differences between groups with a non-Gaussian distribution (Shapiro-Wilk normality test) were determined using Mann-Whitney test and considered significant at $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

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Conflict of interest

The authors state no conflict of interest.

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Figure 1. MC degranulation in lesional skin of EBA patients.

Lesional skin biopsies of EBA patients suffering from inflammatory type of EBA as well as samples of healthy control volunteers were collected and cryosections were stained for Naphthol AS-D chloroacetate esterase. Representative photographs of 5 investigated patients and 4 healthy controls are shown (upper panel). Scale bar = 50 μ m. Regions of interest of these micrographs were shown at a 3fold higher magnification (lower panel). Degranulated MCs are indicated by arrows.

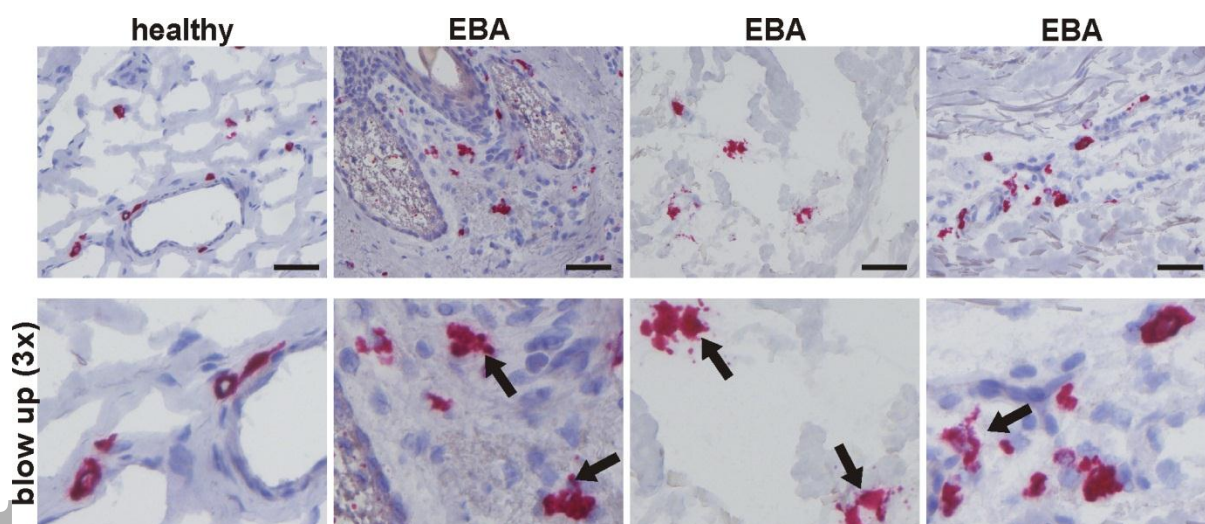


Figure 2. Discrepant susceptibilities of MC-deficient mouse strains to experimental EBA.

(A-C) Mice of the strains (A) W-sh or C57BL/6J (WT), (B) W/W-v or WBB6F₁-Kit^{+/+} (WT), or (C) Cre/iDTR (tg) received repeatedly anti-COL7 IgG or corresponding rabbit control IgG (ctrl) and were scored for clinical symptoms. Prior to antibody administration, Cre/iDTR mice were treated 4 times in a weekly interval either with DT or PBS. Data represent medians with interquartile range pooled from two independent experiments, each performed with 3 (ctrl) and 6 (anti-COL7) mice per experimental group, respectively. Significant differences between groups of WT and W-sh mice, littermates and W/W-v mice, and PBS- and DT-treated mice, respectively, in anti-COL7 treated groups were analyzed according to Mann-Whitney-test and are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (D) MC-deficient and MC-sufficient mice were treated with anti-COL7 IgG. Photographs are representative of two independent experiments.

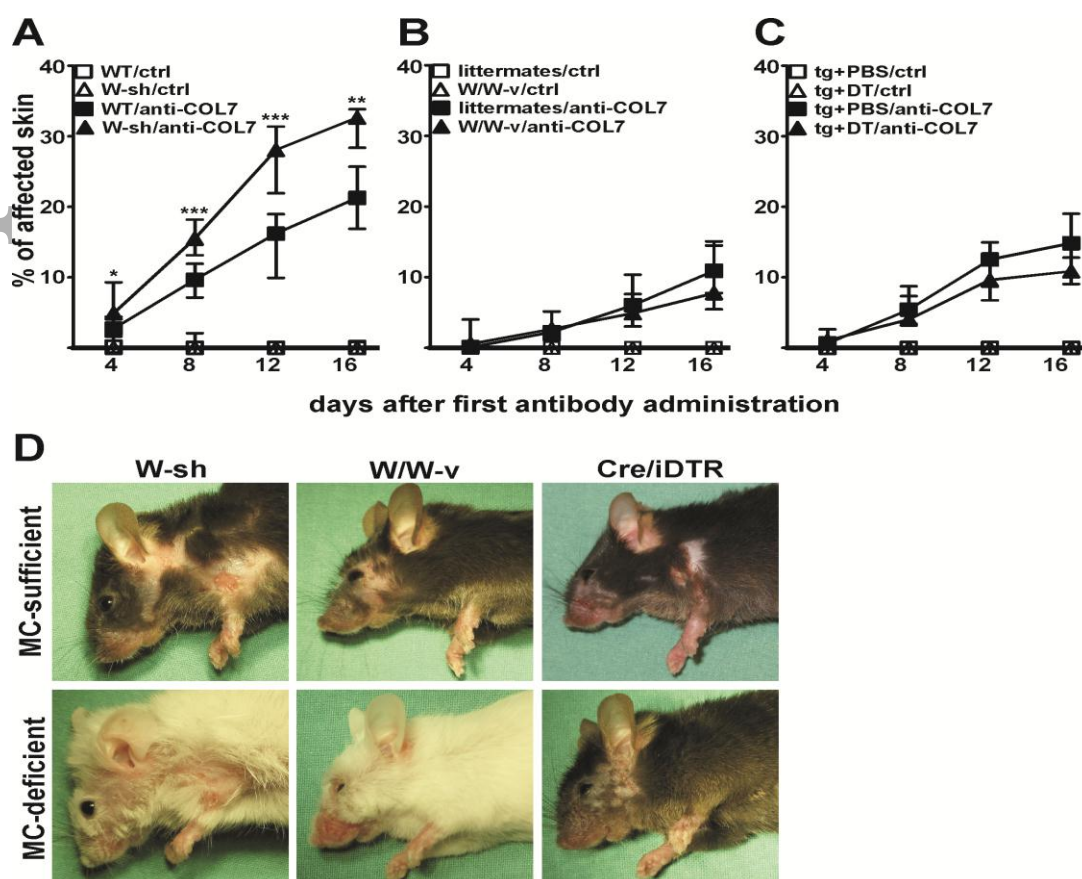


Figure 3. Comparable histopathology in the skin of MC-sufficient and MC-deficient mice in experimental EBA.

W-sh or C57BL/6J (WT), W/W-v or WBB6F₁-Kit^{+/+} (WT), or Cre/iDTR (tg) mice (n= 18) were treated with anti-COL7 IgG or corresponding rabbit control IgG (ctrl) and were sacrificed at day 16. (A) Deposition of IgG (upper panel) and C3 (middle panel) was assessed by immunofluorescence staining and colocalization of both proteins was shown in the overlay (lower panel). Scale bar = 75 μ m. (B) Naphthol AS-D chloroacetate esterase staining of representative skin sections revealed the infiltration of neutrophils into the skin and the splitting from epidermis and dermis independent of the presence of skin mast cells. Scale bar = 100 μ m. Regions of interest within the micrographs of the middle panel are shown with 8fold higher magnification (lower panel). Neutrophils are indicated by asterisks; MCs are indicated by arrows. Images are representative of two independent experiments.

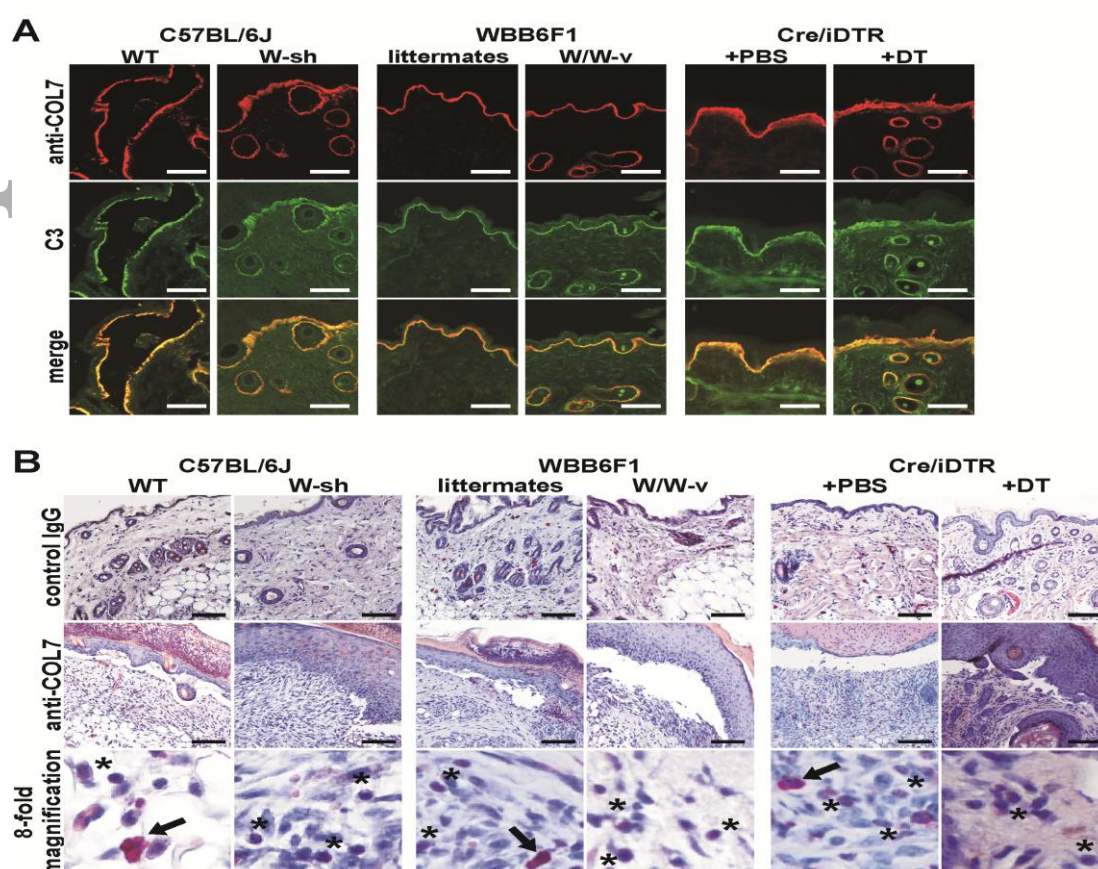
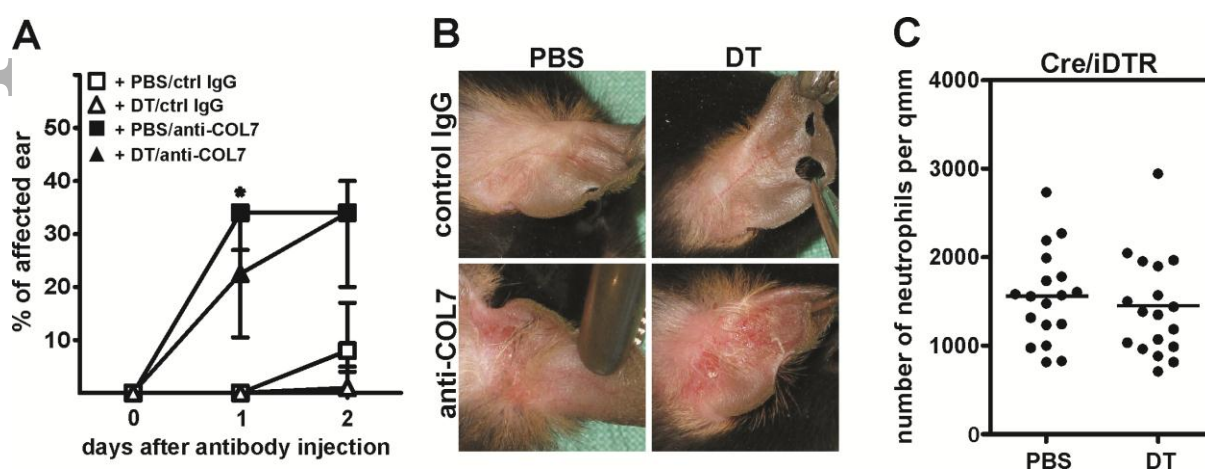


Figure 4. MCs accelerate the pathogenesis in a local model of experimental EBA.

Cre/iDTR mice were pretreated with DT for MC depletion or with PBS (n=20 per group). Both groups received anti-COL7 IgG or corresponding rabbit control IgG (ctrl) intradermally in the ear and were scored for clinical symptoms 24 and 48 hours later (A). Data represent medians with interquartile range and significant differences between PBS- and DT-treated mice of anti-COL7-treated ears are indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ Mann-Whitney-test). (B) Photographs of EBA phenotype are representative for PBS- and DT-treated groups (n=20) 48 hours after antibody administration. (C) Lesional skin tissue was collected at day 2 for paraffin sections and Naphthol AS-D chloroacetate esterase staining was performed. Neutrophils were counted in three mice per experimental group, two lesional skin sections per mouse and three high power fields (400x magnification) per lesional skin section. Data are shown as individual values, and bar represents the arithmetic mean. Data are representative of two independent experiments.



Supplemental information

Supplemental Materials and Methods

Flow Cytometry

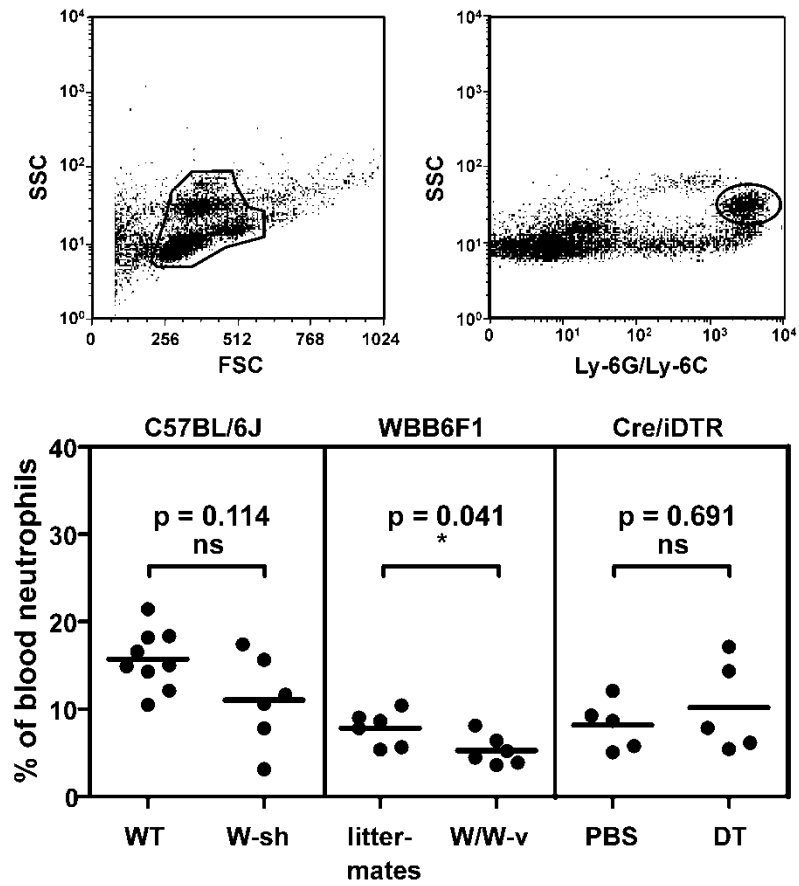
Murine citrate blood was diluted 1/10 in lysis buffer (100 mg KHCO₃, 830 mg NH₄Cl, 180 µL 5% EDTA, diluted in 80 mL deionized water) and incubated for 5 min at room temperature for red blood cell lysis. Antibodies have been diluted in azide-PBS/3% normal human serum. After washing with PBS/0.1% BSA cells were incubated with unconjugated monoclonal rat anti-murine Ly-6G/Ly-6C (IgG2b; clone NIMP-R14; Santa Cruz Biotechnology, Heidelberg, Germany) for 30 min on ice, washed with PBS and incubated with AlexaFluor488-conjugated polyclonal goat anti-rat IgG (Invitrogen, Darmstadt, Germany) for 20 min on ice. Cells were washed, resuspended in azide-PBS and the mean fluorescence intensity of 10,000 cells was measured using FACS Calibur flow cytometer (BD Bioscience). Cells were analyzed on a lymphocyte-sized cell gate and obtained data were evaluated using FCS Express Flow Cytometry software (De Novo, Los Angeles, CA). Nonspecific rat IgG (clone RTK2758) was used as negative control.

Quantification of extravasated Evans Blue dye

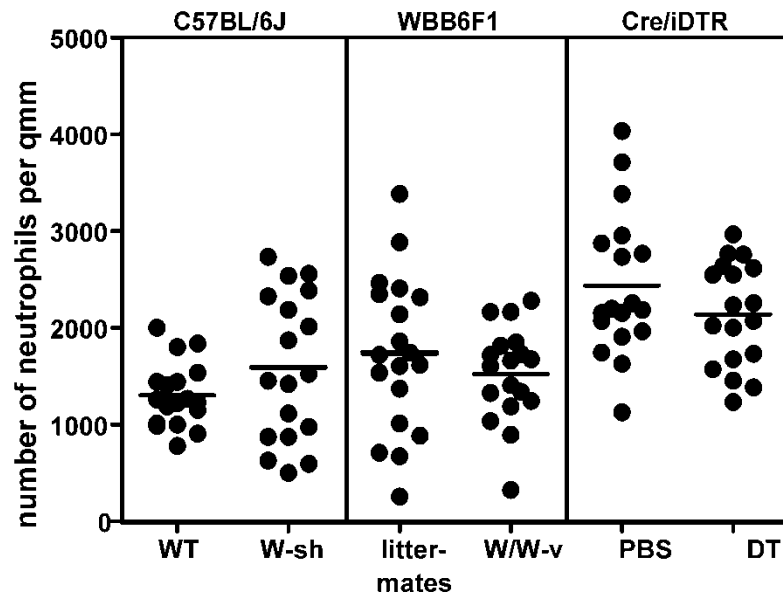
Collected ear samples were dried for 2 days at 60°C followed by extraction of the blue dye with formamide for additional 2 days. For quantification, extinction of extracted dye was determined with the help of a photometer and an appropriate standard curve.

Supplemental figures

A



B

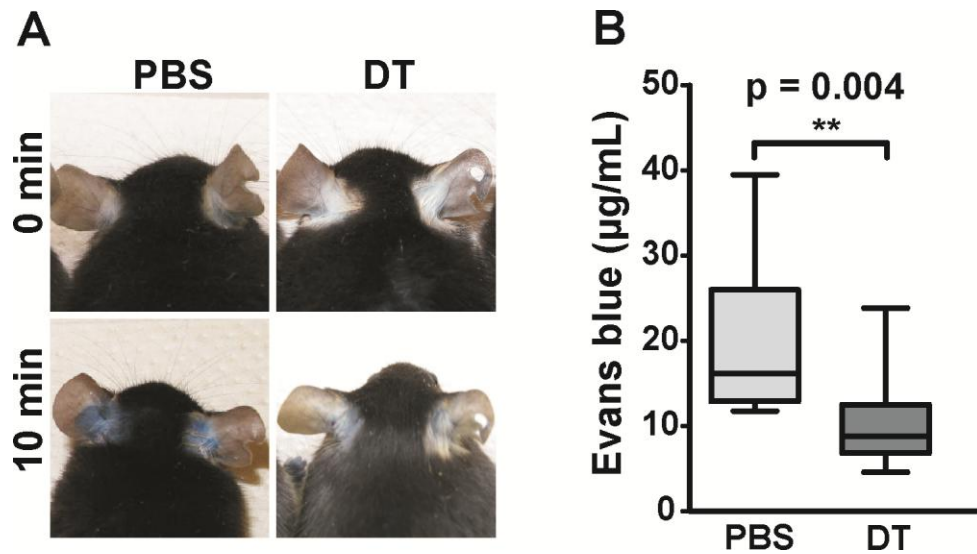


Supplementary Figure S1:

Decreased percentage of blood neutrophils in MC-deficient W/W-v mice but comparable numbers of infiltrated neutrophils in lesional skin of MC-deficient and sufficient mice

Systemic EBA was induced in MC-sufficient and MC-deficient mice of the W-sh, W/W-v and Cre/iDTR strain. (A) Blood samples were collected before first antibody administration and percentage of neutrophils in whole blood was determined by flow cytometry using the NIMP-R14 antibody as neutrophil marker (Ly-6G/Ly-6C). Upper panel shows gating strategy. Results are shown in the lower panel as individual values, bar represents arithmetic mean, and derived from two independent experiments for each strain. Significant differences as determined by Mann-Whitney-test between MC-sufficient and MC-deficient groups are indicated (ns, not significant; *, $p < 0.05$).

(B) Lesional skin tissue was collected at day 16 for paraffin sections and Naphthol AS-D chloroacetate esterase staining was performed. Neutrophils were counted in three mice per experimental group, two lesional skin sections per mouse and three high power fields (400x magnification) per lesional skin section. Data represent individual values with the bar as arithmetic mean. No significant differences between MC-sufficient and MC-deficient mice per mouse strain were determined (Mann-Whitney test).



Supplementary Figure S2:

Edema formation in the local model of experimental EBA depends on MCs

Cre/iDTR mice were pretreated with DT for MC depletion or with PBS. Both groups received Evans blue i.v. followed by intradermal administration of anti-COL7 IgG in the left or corresponding rabbit control IgG in the right ear. Extravasation was determined 10 min after antibody application. (A) Photographs are representative for PBS- (n=17) and DT-treated (n=12) groups. (B) In tissue extravasated Evans blue dye of antibody-treated animals was quantified and presented as box plots (whiskers: Min&Max) of PBS- (n=9) and DT-treated (n=7) mice. ** $p < 0.01$ Mann-Whitney test