

Mast Cells Are Key Promoters of Contact Allergy that Mediate the Adjuvant Effects of Haptens

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SUMMARY

A prominent feature of sensitizing environmental compounds that cause allergic contact dermatitis is the rapid induction of an innate inflammatory response that seems to provide danger signals for efficient T cell priming. We generated mouse models of mast cell deficiency, mast cell-specific gene inactivation, and mast cell reporter mice for intravital imaging and showed that these adjuvant effects of contact allergens are mediated by mast cells and histamine. Mast cell deficiency resulted in impaired emigration of skin DCs to the lymph node and contact hypersensitivity was dramatically reduced in the absence of mast cells. In addition, mast cell-specific inactivation of the *Il10* gene did not reveal any role for mast cell-derived IL-10 in the regulation of contact allergy. Collectively, we demonstrate that mast cells are essential promoters of contact hypersensitivity, thereby highlighting their potential to promote immune responses to antigens entering via the skin.

INTRODUCTION

Contact allergy is a common skin disease of high socioeconomic relevance caused by delayed type hypersensitivity (DTH) responses to environmental antigens (reviewed for example in Grabbe and Schwarz, 1998; Belsito, 1999). An important group of contact allergens are small organic compounds that rapidly penetrate into the skin and bind to proteins in the dermis, a process called “haptization” that renders self-proteins antigenic. Skin resident DCs take up the modified proteins and prime allergen-specific T cells in the skin-draining lymph nodes (LNs), a process called “sensitization.” Upon a second antigen encounter, these T cells rapidly accumulate in allergen-exposed skin where they release proinflammatory cytokines and destroy

allergen-loaded cells by cytotoxic activity (Bour et al., 1995; Wang et al., 2000). A widely used animal model of human contact allergy, contact hypersensitivity (CHS), is the DTH response to small organic haptens with potent sensitizing capacity in the mouse (Grabbe and Schwarz, 1998). Mice are usually sensitized on the shaved trunk, and sensitization is followed by challenge (also referred to as “elicitation”) on the ear several days later and monitoring of ear skin inflammation thereafter (Grabbe and Schwarz, 1998). Evidence that the sensitizing potential of a compound depends on its capacity to provide danger signals has accumulated over the past years (Grabbe et al., 1996; Vocanson et al., 2009; Martin and Jakob, 2008). Haptens used to induce CHS rapidly evoke a local inflammatory response within the first hours after administration (Enk and Katz, 1992). This early innate immune response provides adjuvant effects that promote the induction of the subsequent allergen-specific T cell response (Bonneville et al., 2007). The skin-based cell types that initially sense the haptens and mediate their adjuvant effects remain to be identified. Because mast cells (MCs) are capable of rapidly releasing proinflammatory mediators, they seem to be particularly promising candidates for this task.

MCs are best known as effector cells of IgE-mediated allergic responses, but over the past years important functions of MCs in innate and adaptive immunity, pathogen defense, and autoimmune diseases have been reported (reviewed in Abraham and St John, 2010; Dawicki and Marshall, 2007; Marshall, 2004; Galli et al., 2005; McNeil et al., 2007). The role of MCs in CHS has been considered controversial with several studies reporting MC dependency of CHS (Biedermann et al., 2000; van Loveren et al., 1983), whereas others described undiminished CHS under conditions of MC deficiency (Galli and Hammel, 1984; Mekori and Galli, 1985). Recently, Grimbaldston et al. (2007) even reported enhanced CHS in MC-deficient mice and concluded that MCs suppress contact dermatitis. All these studies, because of the lack of better models, relied on mice carrying mutations of the MC growth factor SCF or its receptor Kit to obtain various degrees of MC deficiency. SCF or Kit mutant mice have been widely used for the investigation of MC functions in vivo over the past 30 years (Galli et al., 2008; Grimbaldston et al.,

2005). However, the interpretation of results obtained with these models has to take into account that the mutant mice feature complex alterations of their immune system, which go beyond the lack of MCs (Nigrovic et al., 2008). Therefore, alternative models of MC deficiency are needed for exploring roles of MCs in immune responses in vivo. Promoting functions of MCs in CHS were implied by experiments in IgE-deficient mice and in animals with defective Fc ϵ R signaling (Bryce et al., 2004, Kobayashi et al., 2010).

Based on our MC-specific Cre transgenic line (Scholten et al., 2008), we have now developed mouse models of inducible or constitutive deficiency for connective tissue type MCs, in which the immune system is not altered except for effects of MC deficiency. Analysis of CHS in these animals reveals that selective MC deficiency results in an almost complete abrogation of the allergic T cell response. We also show that the early innate immune response induced by the adjuvant effects of the hapten is dependent on MCs. Collectively, we demonstrate that MCs promote CHS and question current views on the immunosuppressive potential of these cells (Galli et al., 2008). Our findings highlight the potential of MCs to stimulate adaptive immune responses to antigens entering via the skin.

RESULTS

Mouse Models of Inducible or Constitutive MC Deficiency

In order to investigate MC functions in vivo, we generated mouse models of inducible or constitutive MC deficiency, which were based on our MC-specific Cre transgenic line, *Mcpt5-Cre* (Scholten et al., 2008). As shown by a cross to the Cre excision reporter line *R26Y* (Srinivas et al., 2001), *Mcpt5-Cre* mice delete *loxP*-flanked genomic DNA with high efficiency in MCs, whereas no deletion occurs in other cell types in the peritoneal cavity or spleen (Scholten et al., 2008). In ear skin cell suspensions, 97.5% of MCs expressed EYFP (Figure S1A available online) and, conversely, 97.3% of EYFP⁺ cells were MCs, ruling out Cre activity in a substantial number of non-MCs of the skin. Expression of the reporter did not occur in blood leukocytes except for a small fraction of NK cells (Figure S1B).

Mcpt5-Cre mice were bred to the *iDTR* line (Buch et al., 2005), which expresses a simian diphtheria toxin (DT) receptor selectively in those cells, which delete a *loxP*-flanked stop element. Because WT mouse cells are resistant to DT, only MCs were expected to be sensitive to DT-induced cell death in *Mcpt5-Cre⁺iDTR⁺* offspring. Indeed, a single i.p. injection of DT resulted in an almost complete loss (98.7%) of peritoneal MCs but not skin MCs 24 hr after DT injection (not shown). Four successive i.p. injections of DT efficiently depleted peritoneal (Figure 1A) but also abdominal skin MCs (Figure 1B). However, the depletion of MCs in ear skin, which contains two times more MCs than abdominal skin in C57BL/6 mice, was less efficient (Figure 1B). Because our intention was to analyze local immune responses of ear skin, we tested whether MC depletion could be improved by local administration of DT. Two successive s.c. injections of DT at the base of the ear pinna resulted in a reduction of ear MC numbers to 11.2% (Figure 1B). Combined systemic (4 $\times$ i.p.) and local (2 $\times$ s.c.) treatment depleted 97.5% of MCs in the ear and was routinely used for CHS experiments

(Figure 1B). One week after the last i.p. injection, no significant alterations in the numbers of other cell types were detected (Figure 1C and Figure S2). No significant recovery of MC populations was observed 3 weeks after depletion under steady state conditions or within 1 week under inflammatory conditions (Figure S3). Analysis of small intestine (not shown) and stomach (Figure S4) of DT-injected *Mcpt5-Cre⁺iDTR⁺* mice demonstrated that (subepithelial) connective tissue type MCs but not (intraepithelial) mucosal MCs were efficiently depleted, most likely reflecting a lack of *Mcpt5-Cre* transgene expression in MCs of the mucosal type.

For generation of a model of constitutive MC deficiency, *Mcpt5-Cre* mice were crossed to the *R-DTA* line (Voehringer et al., 2008). In these mice, cells that have deleted a *loxP*-flanked stop cassette express diphtheria toxin A (DTA) and thereby kill themselves. As expected, *Mcpt5-Cre⁺R-DTA⁺* mice showed a significant reduction of MC numbers in peritoneum and skin (Figure 1D).

In summary, *Mcpt5-Cre iDTR* and *Mcpt5-Cre R-DTA* mice represent useful models of inducible or constitutive deficiency of connective tissue type MCs that, in contrast to the various *Kit* mutant lines used to date, are characterized by an otherwise normal immune system.

Reduced CHS Responses in MC-Deficient Mice

In order to investigate the role of MCs in the CHS response to haptens, we epicutaneously sensitized MC-depleted *Mcpt5-Cre⁺iDTR⁺* mice as well as DT-injected *Cre⁺iDTR⁺* littermate and WT control mice at the back and challenged them 6 days later on the ear. The subsequent ear swelling was quantified as a measure of the inflammatory response. The CHS response to DNFB was significantly reduced in MC-depleted *Mcpt5-Cre⁺iDTR⁺* mice in comparison to the controls (Figure 2A). In constitutively MC-deficient *Mcpt5-Cre⁺R-DTA⁺* mice, the ear thickness at 24 hr after DNFB challenge was found reduced to 50% compared to controls (Figure 2B). In order to exclude that our finding of reduced CHS to DNFB reflected a specific property of this allergen, we investigated the response to an additional standard hapten, FITC. As shown in Figure 2C, the CHS response to FITC was also strongly reduced in MC-depleted *Mcpt5-Cre⁺iDTR⁺* mice versus DT-injected controls.

The diminished response to DNFB in MC-depleted mice was reflected by a reduced infiltration of CD45⁺ leukocytes into the ear 24 hr after DNFB challenge (Figure 2D). In particular, the numbers of T cells were significantly lower in MC-deficient mice versus controls. The relative number of neutrophils was not different between the groups indicating approximately half the absolute number of neutrophils in the MC-deficient group.

By injection of DT only at the base of the ear pinna, ears can be locally depleted of MCs (Figure 1B) leaving MCs at other locations unaffected. This offers the possibility of sensitization on the MC-proficient back followed by challenge on the MC-depleted ear. As shown in Figure 2E, the response to DNFB was significantly reduced under these conditions. However, the reduction was not as pronounced as in completely MC-deficient mice, indicating that MCs contribute to both, the sensitization and the elicitation phase of CHS.

Collectively, our results show that MCs are essential for the induction of CHS to DNFB and FITC.

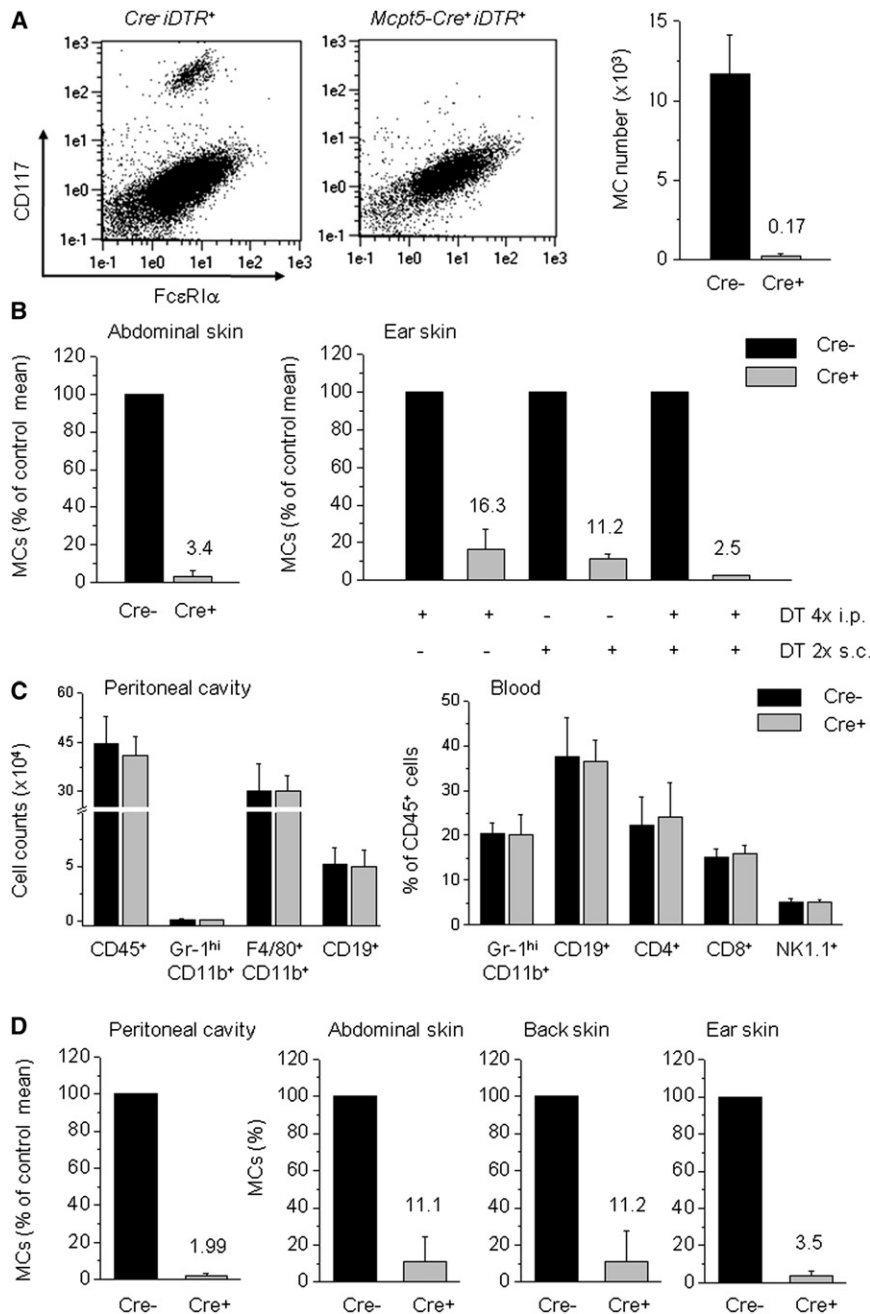


Figure 1. Efficient DT-Mediated MC Depletion in *Mcpt5-Cre iDTR* Mice and MC Deficiency in *Mcpt5-Cre R-DTA* Mice

(A) Mean peritoneal MC numbers $\pm$ SD in *Mcpt5-Cre+ iDTR+* mice (n = 4) and *Cre-* controls (n = 4) after four i.p. injections of DT. (B) Mean MC numbers $\pm$ SD of *Mcpt5-Cre+ iDTR+* mice and *Cre-* controls in abdominal skin after four DT injections i.p. (left) and ear skin after combined systemic and local DT-treatment as indicated. MCs were identified by Giemsa-staining on tissue sections; n = 5 for all groups. (C) Peritoneal and blood leukocyte subsets determined by FACS in *Mcpt5-Cre+ iDTR+* mice (n = 5) and *Cre-* controls (n = 4) after four i.p. plus two s.c. injections of DT; mean $\pm$ SD (D) Mean MC numbers $\pm$ SD of 8-week-old *Mcpt5-Cre+ R-DTA+* (n = 7) and *Cre-* control mice (n = 12) in peritoneal cavity (FACS) and skin (histology).

the deletion was efficient in MCs and was not detected outside the MC lineage (Table S1).

CHS to DNFB was induced in *Mcpt5-Cre+ Il10^{fl/fl}* mice by the exact same protocol as in Grimaldeston et al. (2007) but was found unchanged in comparison to controls, whereas *Il10^{-/-}* mice (Kühn et al., 1993) displayed enhanced CHS as expected (Berg et al., 1995) (Figure 2F).

The finding that CHS is not enhanced in MC-specific *Il10* deficient mice is in accordance with the observation that induced MC deficiency results in a major reduction, but not an increase, of the CHS response. Thus, the effect MCs exert on CHS is stimulatory and not suppressive.

Contact Allergens Induce a MC-Dependent Immediate Response of the Skin Vasculature

In order to address the mechanisms of the stimulatory effect MCs exert on CHS, we directly visualized MCs and blood vessels in untreated and DNFB-treated ear skin by intravital 2-photon microscopy. Analysis of untreated ears of *Mcpt5-Cre+ R26Y+* MC-reporter mice

IL-10 from MCs Is Not Relevant for Suppression of CHS

Grimaldeston et al. (2007) reported that MCs suppress contact allergy by provision of IL-10. We generated mice with a selective inactivation of the *Il10* gene in MCs by crossing the *Mcpt5-Cre* line to *Il10^{fl/fl}* mice (Roers et al., 2004). Efficiency of *Il10* gene inactivation in *Mcpt5-Cre+ Il10^{fl/fl}* MCs was demonstrated by single-target amplification of the *Il10* locus of single FACS-sorted peritoneal or skin MCs or non-MCs. The fragment amplified from MCs always represented the deleted but never the loxP-flanked situation, whereas the opposite result was obtained from non-MCs or from all *Cre-* control cells. Therefore,

revealed that a large fraction of MCs was located in close proximity to blood vessels and displayed an elongated or spindle-like morphology partially oriented in parallel to the vessel axis (Figures 3A and 3B and Movie S1). When mice were challenged by DNFB via the ear skin during the ongoing intravital observation, we noticed a dramatic and rapid increase in blood vessel diameters within minutes after DNFB (Figures 3C and 3D; Movies S2A and S2B), which lasted several hours. In addition, we found that the intravascular dye leaked out of the vessel lumen in DNFB- but not vehicle-treated ears, indicating increased vessel permeability (Figures 3E and 3F, Movie S3, and Movie S4). This leakage

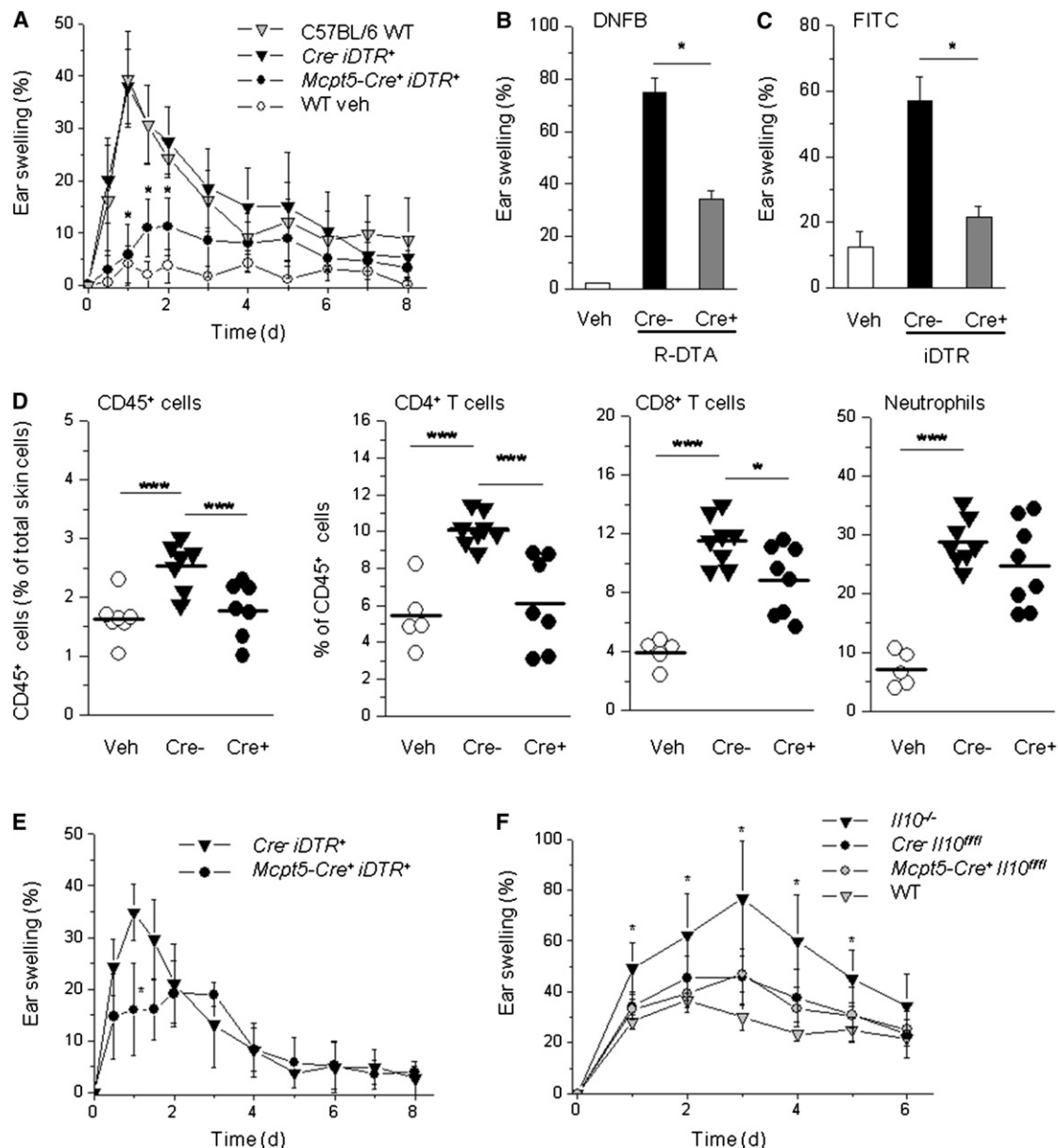


Figure 2. The CHS Response Is Dependent on MCs and Is Not Counterregulated by MC-Derived IL-10

(A) CHS to DNFB in MC-depleted *Mcpt5-Cre*⁺*iDTR*⁺ mice (n = 8), DT-injected *Cre*⁻ controls (n = 8), untreated WT mice (n = 5), and vehicle (veh) controls (n = 3); mean ± SD; *p < 0.005.

(B) CHS to DNFB in *Mcpt5-Cre*⁺ *R-DTA*⁺ mice (n = 5), *Cre*⁻ controls (n = 5), and vehicle controls (n = 3). Mean ear swelling ± SD 24 hr after challenge is shown; *p < 0.005.

(C) CHS to FITC in MC-depleted *Mcpt5-Cre*⁺*iDTR*⁺ mice (n = 4), *Cre*⁻ controls (n = 4), and vehicle controls (n = 3). Mean ear swelling ± SD 24 hr after challenge is shown; *p < 0.005.

(D) FACS analysis of ear skin cell suspensions generated 24 hr after DNFB challenge from MC-depleted *Mcpt5-Cre*⁺*iDTR*⁺ (n = 8), DT-injected *Cre*⁻ control (n = 8), and vehicle-treated (n = 5) *Cre*⁻ mice; ***p < 0.005, *p < 0.05.

(E) CHS to DNFB in *Mcpt5-Cre*⁺*iDTR*⁺ mice and *Cre*⁻ controls injected with DT at the base of the ear pinna but not systemically. Mice were sensitized on the MC-proficient back and challenged at the MC-depleted ear; mean ± SD, *p < 0.05, n = 4 per group.

(F) CHS to DNFB in WT (n = 3), *Il10*^{-/-} (n = 7), *Mcpt5-Cre*⁺*Il10*^{fl/fl} (n = 5) and *Cre*⁻*Il10*^{fl/fl} littermate controls (n = 14). Protocol for CHS induction exactly as in Grimbaldston et al. (2007); mean ± SD, *p < 0.005.

started within minutes after DNFB. Time-lapse studies of untreated ear skin revealed that MCs were immobile cells with little motion on the cell periphery (Movie S2A). Also after DNFB

challenge, we did not observe significant MC migration or lymph vessel entry within an observation period of 12 hr after DNFB. However, allergen challenge induced changes of MC

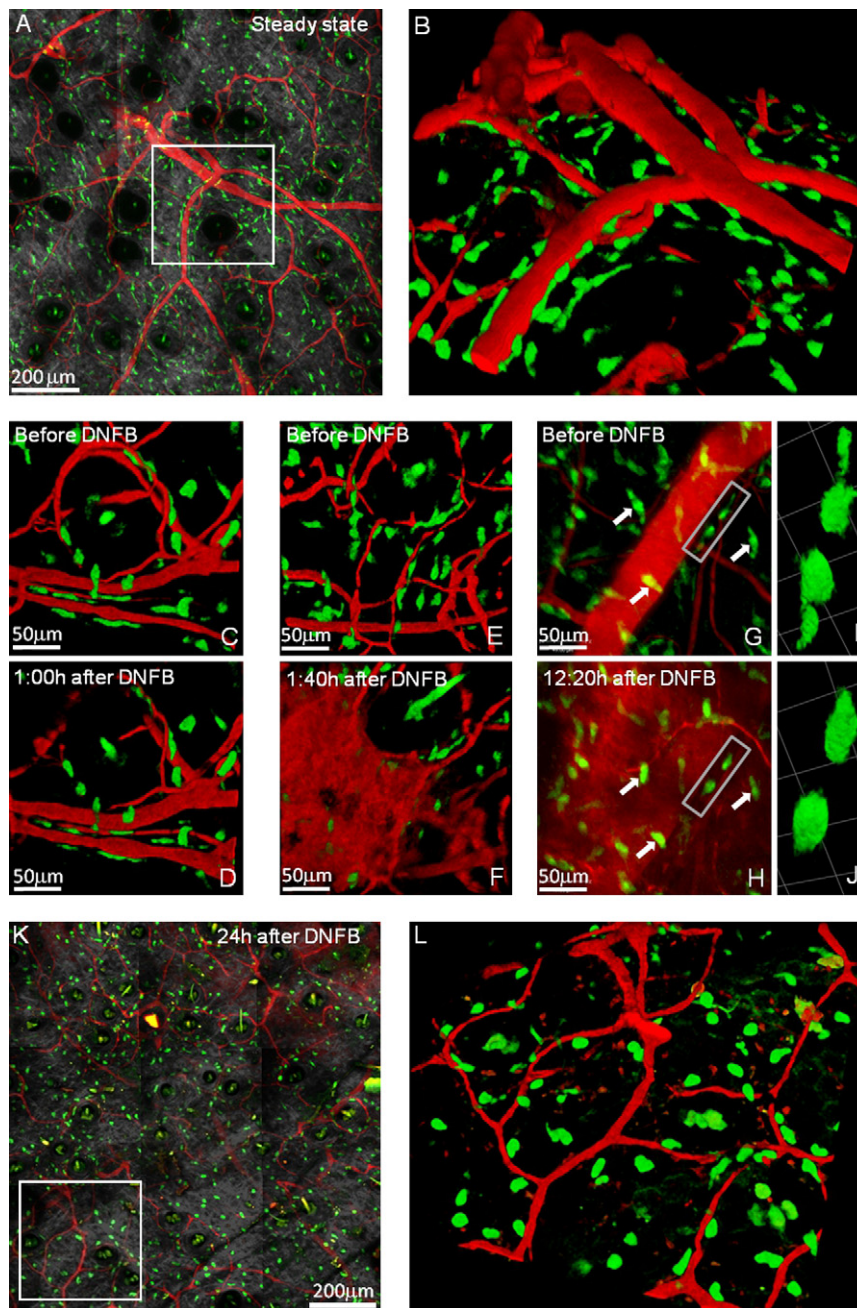


Figure 3. Intravital Two-Photon Microscopy of *Mcpt5-Cre⁺R26Y⁺* Mice Reveals that Ear Skin MCs and Blood Vessels Respond to DNFB

Array of 3×3 maximum intensity projections (A) and surface-rendered Z stack (B, [Movie S1](#)) showing distribution and morphology of MCs (green), blood vessels (i.v.-injected red Qdots), and collagen fibers (gray) of untreated skin. DNFB challenge in sensitized mice induces vasodilation (C and D; [Movie S2](#)) and Qdot leakage (E and F; [Movie S3](#) and [Movie S4](#)), as well as changes in MC morphology (G–L, [Movie S5](#) and [Movie S6](#)); maximum intensity projections (G and H), surface-rendered Z stacks (I and J, blow-ups from G and H, respectively), and array of 3×3 maximum intensity projections (K).

Cre⁻iDTR⁺ control mice reproduced the dilatation of blood vessels described above. However, in *Mcpt5-Cre⁺iDTR⁺* animals the dilatation of larger ($\geq 18 \mu\text{m}$) vessels was significantly reduced compared to the controls, whereas smaller ($<18 \mu\text{m}$) vessels showed undiminished dilatation ([Figure 4A](#)). A quantitative comparison of the DNFB-induced leakage of the intravascular dye in MC-depleted versus control mice proved difficult because of local variation of this phenomenon. Therefore, we relied on ear thickness measurements for quantification of the tissue edema. In our hands, DNFB caused an immediate ear swelling that peaked after 2 hr ([Figure S5](#)). In sensitized mice, this first peak was followed by a second increase in ear thickness peaking at 24 hr ([Figure S5](#)). Also urushiol, TNCB, FITC, and oxazolone induced an early ear swelling with a maximum between 1 and 2.5 hr after exposure ([Figure S5](#)) preceding the later T cell-mediated allergic inflammation. Thus, an early inflammatory response is a common theme in sensitization to haptens. As shown in [Figure 4B](#), the immediate ear swelling response in sensitized and non-sensitized control mice was almost

morphology. Within hours after DNFB exposure, MCs gave up the branched and spindle-like appearance to adopt more globular shapes ([Figures 3G–3L](#), [Movie S5](#), and [Movie S6](#)). Initially, only few MCs underwent these changes while most MCs were affected after 24 hr. This phenomenon did not occur after treatment with vehicle alone (not shown).

Because MCs are known to induce rapid vascular responses by releasing prestored mediators, we examined whether the immediate response of the vasculature to contact allergens was mediated by MCs. Intravital imaging allowed direct measurement of diameters of individual skin blood vessels before and after allergen administration. DNFB-treated

completely abrogated in the MC-depleted animals. The MC-dependence of this response was confirmed in *Mcpt5-Cre⁺iDTR⁺* mice after local MC-depletion in ear tissue only (data not shown) and also in MC-deficient *Mcpt5-Cre⁺R-DTA⁺* ([Figure 4C](#)), *Kit^{W/W⁻}*, and *Kit^{W/W⁻}EPO-tg* mice (see below). Like the immediate response to DNFB, also the immediate response to FITC was almost completely abrogated in MC-depleted *Mcpt5-Cre⁺iDTR⁺* mice ([Figure 4D](#)). Thus, a prominent effect of haptens is the immediate induction of a vascular response that is dependent on MCs and that occurs independent of prior sensitization. The MCs act as amplifier of the dilatation of larger vessels and mediate a rapid increase in vessel permeability.

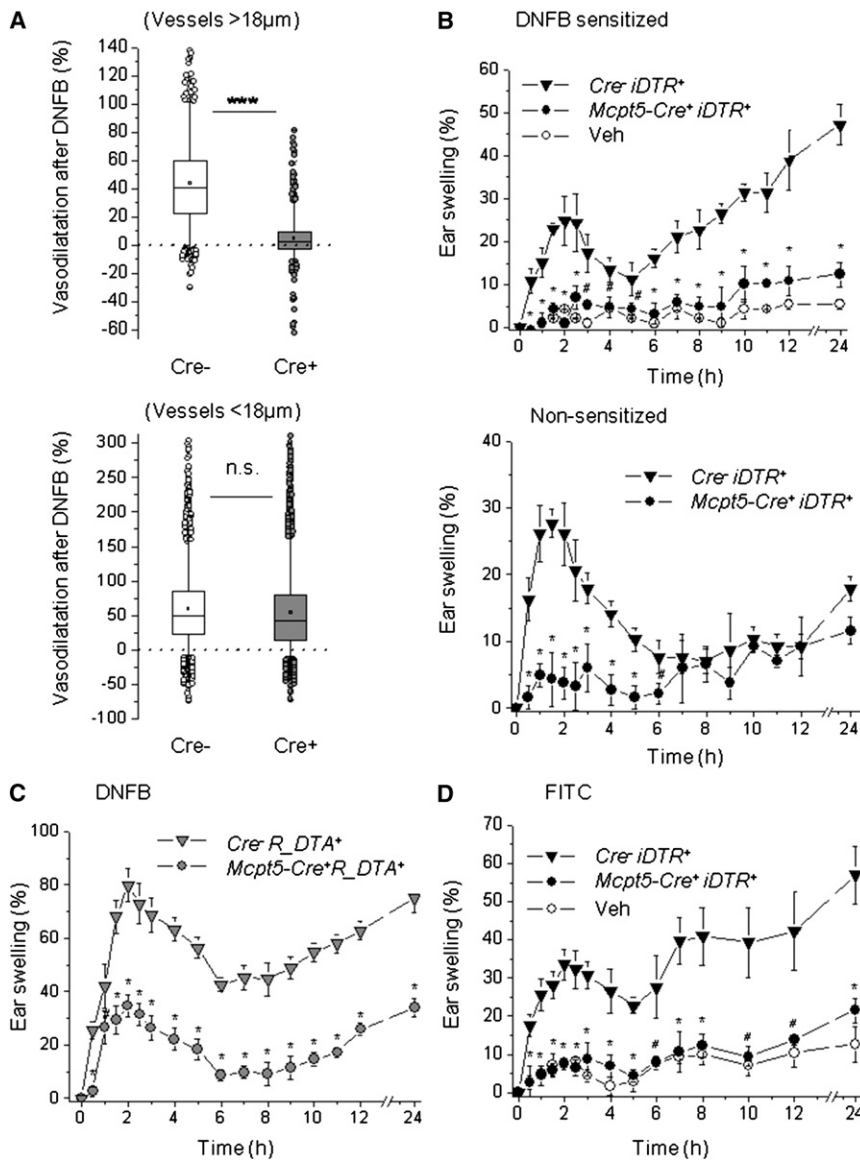


Figure 4. The Early Innate Response to Haptens Is MC Dependent

(A) Increase in blood vessel diameter in ear skin of sensitized *Mcpt5-Cre⁺iDTR⁺* mice and DT-injected *Cre⁻* controls as determined by intravital two-photon microscopy 1 hr after DNFB challenge compared to prechallenge diameter. Pooled data from five *Cre⁺* and five *Cre⁻* mice; ****p* < 0.005.

(B) Ear swelling response to DNFB in sensitized or non-sensitized MC-depleted *Mcpt5-Cre⁺iDTR⁺* mice (*n* = 5), DT-injected *Cre⁻* controls (*n* = 5), and vehicle (veh) controls (*n* = 3); mean ± SD, **p* < 0.005, #*p* < 0.05.

(C) Ear swelling response to DNFB in *Mcpt5-Cre⁺R- DTA⁺* mice (*n* = 5) and *Cre⁻* controls (*n* = 5); mean ± SD, **p* < 0.005, #*p* < 0.05.

(D) Ear swelling response to FITC in *Mcpt5-Cre⁺iDTR⁺* mice (*n* = 4), *Cre⁻* controls (*n* = 4) and vehicle controls (*n* = 4); mean ± SD, **p* < 0.005, #*p* < 0.05.

diate response to FITC was greatly diminished in pyrilamine pretreated animals (Figure 5C).

Interestingly, cromolyn and pyrilamine not only blocked the immediate response but also significantly reduced the subsequent increase in ear thickness in sensitized mice that reflected the adaptive CHS response (Figures 5A–5C). This observation prompted the question whether histamine action during the first hours after DNFB challenge was important for the T cell-mediated CHS response. To address this question, we used a modified CHS protocol that relies on a single DNFB administration (challenge dose of 20 μl, 0.2% DNFB) on the ear without prior sensitization (Saint-Mezard et al., 2003). In naive animals, DNFB painting of the ear results in sensitization but because of persistence of the allergen in the tissue, DNFB painting also

Early Histamine Effects Mediate the Rapid Response of the Vasculature after DNFB Challenge but Also Affect the Subsequent CHS Response

Because the DNFB-induced early response of the vasculature was dependent on MCs, we quantified MC degranulation on Giemsa-stained paraffin sections of ears taken 30 min or 1 hr after DNFB or FITC challenge. As shown in Figure S6, allergen treatment resulted in microscopically evident MC degranulation. We next examined whether MC degranulation and histamine are required for the DNFB-induced immediate response of ear skin blood vessels. Therefore, DNFB-sensitized or unsensitized WT mice were pretreated with the MC-stabilizing agent cromolyn or the histamine receptor-1 antagonist pyrilamine 1 hr before DNFB challenge and the ear swelling response to DNFB was quantified. Pretreatment of the animals with cromolyn (Figure 5A) or pyrilamine (Figures 5B and 5D) resulted in a reduced DNFB-induced immediate ear swelling response. Likewise, the im-

mediate response to FITC was greatly diminished in pyrilamine pretreated animals (Figure 5C). Interestingly, cromolyn and pyrilamine not only blocked the immediate response but also significantly reduced the subsequent increase in ear thickness in sensitized mice that reflected the adaptive CHS response (Figures 5A–5C). This observation prompted the question whether histamine action during the first hours after DNFB challenge was important for the T cell-mediated CHS response. To address this question, we used a modified CHS protocol that relies on a single DNFB administration (challenge dose of 20 μl, 0.2% DNFB) on the ear without prior sensitization (Saint-Mezard et al., 2003). In naive animals, DNFB painting of the ear results in sensitization but because of persistence of the allergen in the tissue, DNFB painting also

elicits CHS and an ear swelling response beginning about 48 hr after allergen exposure. This “primary” CHS response is of similar intensity as compared to the response of sensitized mice but occurs 40 hr later. This system offered the possibility to block histamine during the initial allergen contact and investigate the CHS response that ensued without secondary allergen challenge. To exclude that the histamine blockade was long-lasting and directly affected the CHS response 72 hr after DNFB, we demonstrated that pyrilamine effects were no longer detectable after 48 hr. The histamine-mediated immediate response to DNFB was fully restored at this time point (not shown). Pyrilamine injection 1 hr before allergen exposure blocked the immediate ear swelling (as shown above) but also resulted in a significant reduction of the CHS response commencing 48 hr later (Figure 5D).

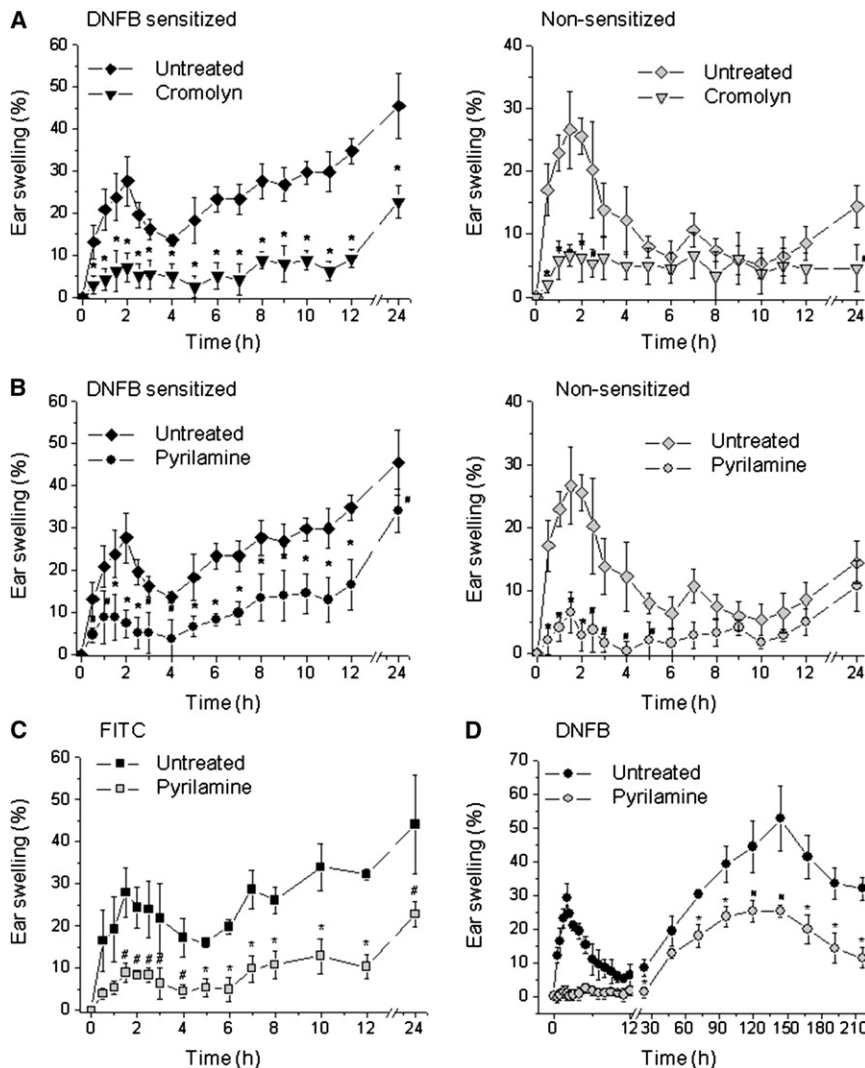


Figure 5. Early Innate Response to Haptens Is Dependent on Histamine

(A–C) Ear swelling response to DNFB or FITC in sensitized or nonsensitized WT mice, untreated or pre-treated with cromolyn (A) or pyrilamine (B and C); $n = 5$ all groups, mean $\pm$ SD, * $p < 0.005$, # $p < 0.05$. (data from the same single experiment are shown in A and B, untreated control groups of sensitized and non-sensitized WT mice are identical in A and B). (D) “Primary” CHS response after a single DNFB administration on the ear (20 μ l, 0.2% DNFB in acetone/oil) in nonsensitized WT mice untreated ($n = 5$) or pretreated with pyrilamine ($n = 5$); mean $\pm$ SD, * $p < 0.01$, # $p < 0.05$.

stimulation of DC migration. Painting of the skin with the contact allergen FITC results in an accumulation of FITC⁺DCs in skin draining LNs, which reflects DC migration from the skin into the LNs (Kripke et al., 1990). In order to determine MC effects on DC migration during the sensitization phase, we treated MC-depleted mice and DT-injected controls with FITC on the back and analyzed the inguinal LNs 48 hr later. Figure 6B shows that the response to FITC resulted in increased LN DC numbers in the control group, similar to the numbers observed after DNFB exposure (Figure 6A). Importantly, total LN cellularity and total DC numbers were reduced and the number of FITC⁺DCs was two times lower in the MC-depleted group as compared to controls (Figure 6B). These results show that MCs have important stimulatory effects on the response of the adaptive immune system to haptens. Stimulation of DC

from degranulating MCs. We further demonstrate that these histamine effects during the first hours after DNFB are of critical importance for the allergic sensitization.

MCs Promote DC Migration and Hypertrophy of Lymph Nodes Draining Contact Allergen-Treated Skin

In order to investigate whether MCs impact on the immune response to DNFB, we sensitized mice on the back and analyzed the inguinal LNs 72 hr later. The LNs were macroscopically enlarged in DNFB-treated mice (data not shown) and contained two times more cells as compared to untreated mice (Figure 6A). In contrast, LNs from MC-depleted *Mcpt5-Cre⁺iDTR⁺* mice displayed a significantly less pronounced DNFB-induced increase in cellularity, that primarily reflected lower numbers of T cells and DCs as compared to DT-injected *Cre⁻* control LNs (Figure 6A).

Sensitization with haptens results in emigration of DCs from the skin to the draining LNs (Kripke et al., 1990). One mechanism through which MC-mediated effects may promote the adaptive immune response against haptens could be the

migration seems to be one mechanism by which MCs promote contact allergy.

Kit Mutant Mice Display Enhanced CHS but Differ in their Early Innate Response to DNFB

Grimbaldeston et al. (2007) reported enhanced CHS to DNFB in the MC-deficient lines *Kit^{W/W-v}* and *Kit^{W-sh/W-sh}*. Also in our hands, these mice displayed enhanced and prolonged ear swelling responses to DNFB as compared to *Kit^{+/+}* controls (Figures 7A and 7B). The immediate ear swelling response to DNFB, which we had found in control but not MC-depleted mice in the *Mcpt5-Cre iDTR* system, was absent in *Kit^{W/W-v}* and in *Kit^{W/W}* mice rescued from early lethality by an EPO transgene (*Kit^{W/W}Epo-tg*, Waskow et al., 2004; Figures 7C and 7D), supporting the MC dependence of this phenomenon. Interestingly, we observed an immediate ear swelling response to DNFB in *Kit^{W-sh/W-sh}* mice (Figure 7E) that was similar to the response of control mice. This response was not due to residual MCs given that we found the MC numbers in ear skin reduced to less than 1% of normal numbers in the 12-week-old *Kit^{W-sh/W-sh}*

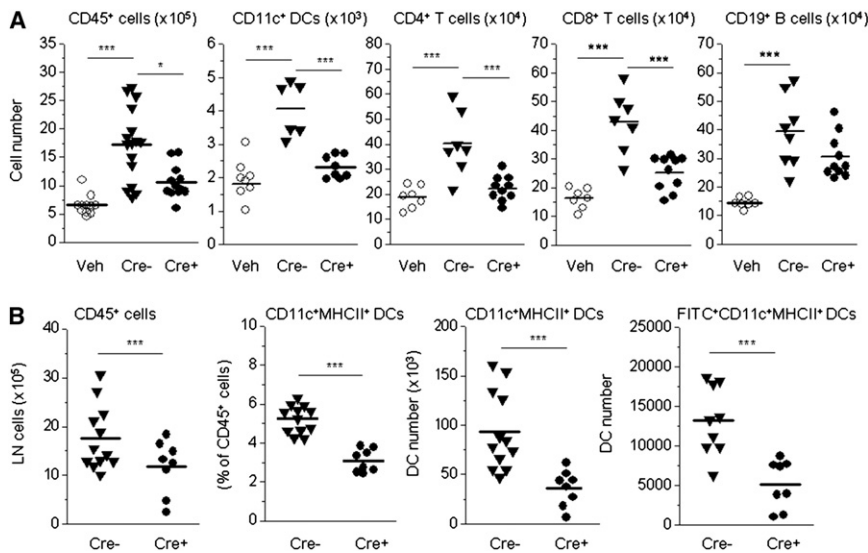


Figure 6. MCs Promote Hapten-Induced LN Hypertrophy and DC Migration

FACS analysis of skin draining LNs.

(A) Numbers of the indicated cellular subsets in inguinal LNs 72 hr after DNFB sensitization at the back of *Mcpt5-Cre⁺iDTR⁺* mice ($n = 8$) and *Cre⁻* controls ($n = 8$) compared to vehicle-treated WT mice ($n = 8$); *** $p < 0.005$, * $p < 0.05$.

(B) *Mcpt5-Cre⁺iDTR⁺* mice ($n = 8$) and *Cre⁻* controls ($n = 12$) were sensitized with FITC on the back. Forty-eight hours later, the number of CD45⁺ cells, CD11c⁺MHCII⁺ DCs and FITC⁺CD11c⁺MHCII⁺ DCs were determined in inguinal LNs; *** $p < 0.005$.

MC-deficient without the requirement for toxin injections (*Mcpt5-Cre R-DTA*).

The question of whether MCs contribute to contact allergy was addressed by numerous studies over the

mice used for the experiments. This result contrasted with the absence of the immediate response in all other MC-deficient or MC-depleted mice analyzed in this study. We thus examined whether the immediate response of *Kit^{W-sh/W-sh}* animals was mediated by histamine. As shown in Figure 7F, the early DNFB response was efficiently suppressed by pretreatment of *Kit^{W-sh/W-sh}* mice with pyrilamine but only mildly reduced by injection of the MC stabilizer cromolyn, suggesting that some other histamine producing cell type could be responsible for the early DNFB response in these mice. Neutrophils were shown to contribute to inflammation in a histamine-dependent fashion (Xu et al., 2006). In order to test, whether the immediate response of *Kit^{W-sh/W-sh}* mice was mediated by neutrophils, we depleted Gr-1-positive cells prior to DNFB painting. This treatment resulted in a significant reduction of the immediate ear swelling response of *Kit^{W-sh/W-sh}* mice, whereas injection of an isotype control antibody had no effect (Figure 7G).

Collectively, we confirmed that the *Kit* mutant lines *Kit^{W/W-v}* and *Kit^{W-sh/W-sh}* show enhanced CHS responses to DNFB. This enhanced immune response, however, is not caused by the MC deficiency of these mice because selective depletion of MCs in our *Mcpt5-Cre⁺iDTR⁺* mice or MC deficiency in the *Mcpt5-Cre⁺R-DTA⁺* animals resulted in a profound reduction of CHS. In contrast to CHS in WT mice, the enhanced CHS in the *Kit^{W/W-v}* line was independent of an early innate response. *Kit^{W-sh/W-sh}* mice mount an abnormal neutrophil- and histamine-dependent early innate response.

DISCUSSION

We have analyzed the role MCs play in contact allergy by using mouse models of MC deficiency and MC-specific gene inactivation either lacking the population of connective tissue type MCs (CTMCs) in all tissues or lacking specific gene products selectively in CTMCs, respectively. Our results clearly demonstrate that MCs are critical for the CHS response to haptens. CHS was reduced in mice selectively depleted of MCs by DT-injection (*Mcpt5-Cre iDTR*) and in mice that were constitutively

past decades, which yielded heterogeneous results. Several reports described undiminished CHS, whereas others found reduced or enhanced CHS in different MC-deficient mouse lines. These discrepant findings may be partially explained by the use of different contact allergens, the use of different protocols for CHS elicitation, quantification of the inflammatory response at different time points, and the mouse models employed.

Mice with compromised SCF-Kit axis have been widely used as models of MC deficiency (Galli et al., 2008). Most of the published literature on in vivo MC functions was based on these models. However, *Kit* is expressed also outside the MC lineage, in particular in hematopoietic stem cells and precursor cells of most lineages. Therefore, *Kit* deficiency results in complex alterations of many hematopoietic compartments. Both, *Kit^{W/W-v}* and *Kit^{W-sh/W-sh}* mice are almost completely MC deficient by the age of 12 weeks. However, the two lines differ dramatically from each other in various immune parameters: *Kit^{W/W-v}* develop macrocytic anemia and neutropenia, display a decrease of intestinal $\gamma\delta$ but an increase in intestinal $\alpha\beta$ T cells, and have a high rate of spontaneous dermatitis. *Kit^{W-sh/W-sh}* mice are characterized by an enlarged spleen with abnormal architecture and increased splenic myeloid cell and megacaryocyte counts, as well as peripheral blood neutrophilia and thrombocytosis. Notably, the lines behaved differently in some disease models (Zhou et al., 2007; Piliponsky et al., 2010). In line with this notion, we show here that the two MC-deficient *Kit* mutant lines displayed fundamentally different innate responsiveness to contact allergens. These differences between the two lines illustrate the alteration of their immune system beyond MC deficiency. In order to demonstrate that a phenotype observed in *Kit* mutants is due to their MC-deficiency, we routinely reconstituted these mice with bone marrow-derived MCs differentiated in vitro (BMMC). Rescue of an observed phenotype by this MC reconstitution was interpreted as proof that the phenotype was due to the loss of MC functions. Using this “MC knock-in” model, Grimaldeston et al. (2007) showed that the exaggerated CHS response of *Kit^{W/W-v}* and *Kit^{W-sh/W-sh}* mice was reduced to normal levels by reconstitution with BMMC from WT but not

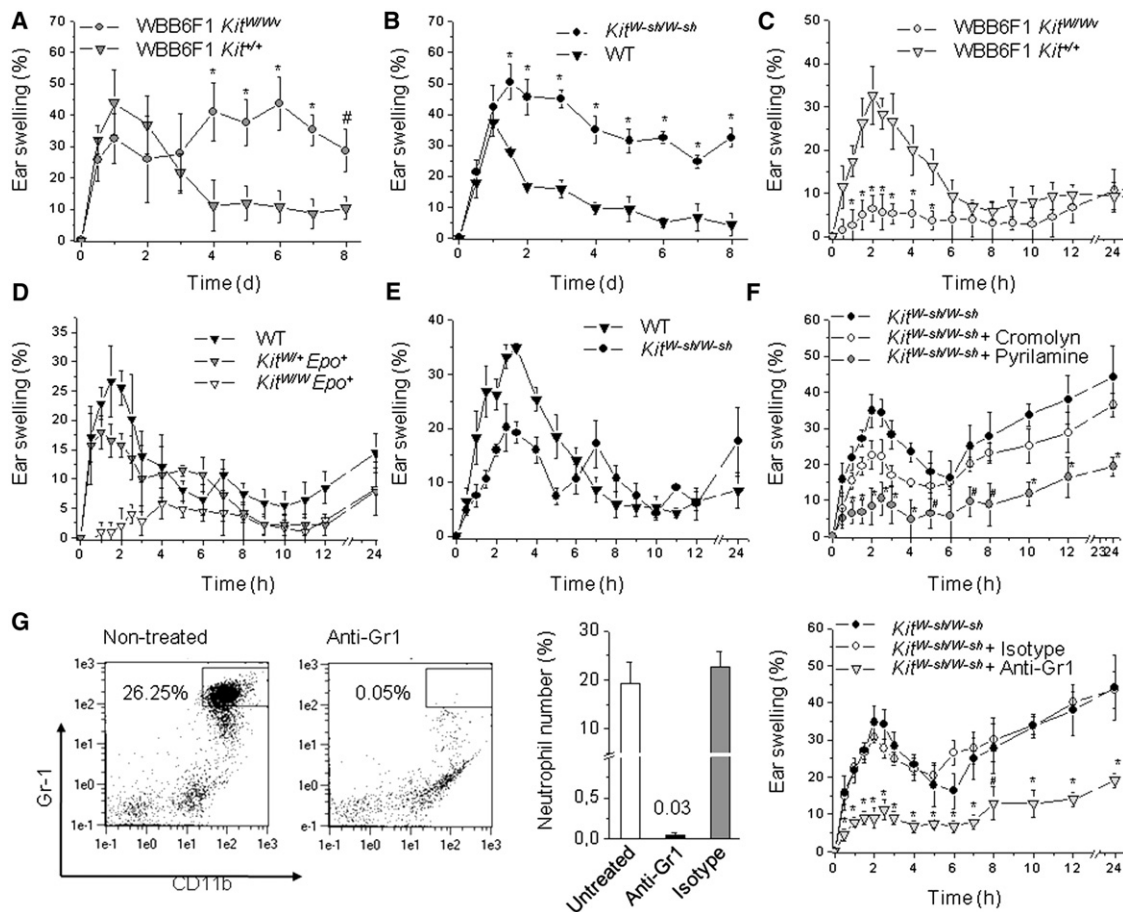


Figure 7. *Kit* Mutant Mice Display Enhanced CHS but Differ in their Early Innate Response to DNFB

(A and B) Ear swelling response to DNFB in sensitized WBB6F1 *Kit^{WVW}* mice (n = 8) and WBB6F1 *Kit^{+/+}* controls (n = 5) or C57BL/6 *Kit^{W-sh/W-sh}* (n = 8) and C57BL/6 WT mice (n = 8); mean $\pm$ SD, *p < 0.005, #p < 0.05.

(C–E) Ear swelling response to DNFB in nonsensitized WBB6F1 *Kit^{WVW}* (n = 8) and WBB6F1 *Kit^{+/+}* controls (n = 5), *Kit^{WVW}Epo^{-tg}* (n = 3), *Kit^{+/+}Epo^{-tg}* (n = 3), and WT controls (n = 4) or C57BL/6 *Kit^{W-sh/W-sh}* (n = 8) and C57BL/6 WT mice (n = 8), *p < 0.005, #p < 0.05.

(F) Ear swelling response to DNFB in sensitized *Kit^{W-sh/W-sh}* mice untreated or pretreated with cromolyn or pyrilamine, n = 5 per group, mean $\pm$ SD; *p < 0.005, #p < 0.05.

(G) Ear swelling response to DNFB in sensitized *Kit^{W-sh/W-sh}* mice untreated or pretreated with an anti-Gr-1 mAb or isotype control, mean $\pm$ SD, n = 4 per group, *p < 0.005, #p < 0.05 (left). The efficiency of neutrophil depletion was determined by FACS analysis of blood leukocytes. Error bars show the mean $\pm$ SD of neutrophil numbers determined as Gr-1^{hi}CD11b⁺ cells, n = 4 (middle and right).

from *Il10^{-/-}* mice and concluded that MCs suppress CHS by provision of IL-10.

We confirmed the enhanced CHS response in the two *Kit* mutant lines. However, CHS was significantly reduced in our *Kit*-independent mouse models of selective MC deficiency, which do not display alterations of the immune system except for the effects of the absence of MCs. We conclude that the exaggerated CHS in *Kit* mutant animals is not caused by the absence of MCs but is related to the *Kit* deficiency. We also demonstrate that mice with an efficient *Cre-loxP*-mediated inactivation of the *Il10* gene in MCs (*Mcp5-Cre Il10^{fl/fl}*) do not show enhanced CHS in response to DNFB. Thus, MCs are important promoters but not suppressors of the CHS response. These results show, that even if a phenotype observed in a *Kit* mutant line can be “repaired” by MC “knock-in,” this does not necessarily prove that the phenotype was caused by the lack of MCs. Collectively, our data show that *Kit* mutant mice can

display alterations of their immune responsiveness that are not related to their MC deficiency. Therefore, in vivo MC functions that have been described on the basis of findings in *Kit* mutant mouse lines, with or without “MC knock-in,” should be re-evaluated with using mouse models that are independent of compromised *Kit* signaling.

In accordance with our results, two reports in mutants with normal *Kit* signaling suggested an important promoting function of MCs in CHS. Lack of IgE in *IgE^{-/-}* mice resulted in reduced CHS responses, which could be restored by exogenous IgE of irrelevant specificity (Bryce et al., 2004). Similarly, CHS was reduced in mice with defective FcεRI signaling (Kobayashi et al., 2010). These key studies suggest that either tonic antigen-nonspecific signals from the IgE-loaded FcεRI may be required for full responsiveness of MCs to contact allergens or, alternatively, that the haptens directly activate MCs via the IgE-FcεRI axis.

An important progress in our understanding of CHS is the realization that contact allergens rapidly induce an inflammatory response of the local innate immune system (Watanabe et al., 2007; Martin et al., 2008). The intensity of this early response was demonstrated to correlate with the severity of the subsequent T cell-mediated CHS response (Bonneville et al., 2007). This led to the interpretation that contact allergens have adjuvant properties that ensure the provision of danger signals for induction of the hapten-specific T cell response. The molecular basis of adjuvant effects of a contact allergen were directly demonstrated in a recent study describing that Ni^{2+} ions ligate and activate human TLR4 and that this TLR-mediated activation of innate immunity is a prerequisite for the allergic sensitization to nickel (Schmidt et al., 2010).

We attempted to clarify the role of MCs in the early innate response to haptens and made use of our EYFP-MC reporter mice (*Mcpt5-Cre R26Y*) to visualize living MCs in their natural environment by intravital 2-photon microscopy. In contrast to other intravital 2-photon imaging approaches, e.g., in LNs, the imaging of MCs in ear skin was practically noninvasive. Imaging of MCs in untreated mice demonstrated high numbers of strikingly elongated or spindle-shaped MCs partly in intimate association with blood vessels. In time-lapse studies we did not observe substantial migratory behavior of MCs within the first 12 hr after DNFB. However, we found that MCs responded to DNFB by changes in morphology. Starting few hours after allergen treatment, increasing numbers of MC contracted to adopt more globular shapes. At 24 hr after DNFB, hardly any MC remained spindle shaped.

Although the biological functions that are reflected by these morphological changes remain unclear, we show that MCs are essential for the early innate response to haptens by using our new MC-deficient mouse lines. Intravital microscopy demonstrated that the DNFB induced dilation of larger skin blood vessels was significantly reduced in MC-depleted mice. This MC-dependent vascular response correlated with the observation that the early ear swelling response to haptens that occurs in WT mice and peaks after 1–2 hr was virtually absent in MC-depleted mice. We also show that this MC-mediated early innate response is dependent on histamine. This finding was unexpected given that an earlier study described enhanced CHS in mice deficient for histidine decarboxylase, which cannot produce histamine (Garaczi et al., 2004). Histamine effects on the immune system are highly complex and have e.g., major impact on T cell differentiation. Therefore, the possibility that the responsiveness of the adaptive immune system is altered in animals that are histamine-deficient throughout ontogeny should be considered.

We show that MCs are essential in the initial activation of an innate response by adjuvant effects of the haptens. This early inflammatory response may promote the T cell response to the hapten by several mechanisms including the induction of DC migration from the hapten-treated skin into the draining LNs. We demonstrated that hapten-induced DC migration was impaired in MC-depleted mice confirming a previous report (Suto et al., 2006). Our findings are in accordance with a recent study demonstrating that MC-activating compounds have potent adjuvant effects in vivo (McLachlan et al., 2008).

Contact allergens differ in their sensitizing potential. Some contact allergens sensitize all or most exposed individuals,

whereas sensitization to weaker allergens occurs only in few individuals and requires multiple exposures. Factors that predispose these few patients to mount an allergic response are unknown but may be due to enhanced MC responsiveness. The difference between potent and weak allergens was linked to their capacity to induce innate local inflammation, and thus, their adjuvant potential, which may reflect their MC-stimulating capacity.

Our results demonstrate that MCs and histamine are critical for induction of contact allergy. This raises the possibility that allergic sensitization in the human could be prevented by antihistamine treatment during exposures to contact allergens.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 *Kit^{W-sh/W-sh}* mice, *Il10^{-/-}* and *Mcpt5-Cre* mice crossed to the *iDTR*, *R-DTA*, *R26Y*, or *Il10^{fl/fl}* lines, as well as *Kit^{W/W-v}* and WT C57BL/6 mice, were housed at the Experimental Centre at the University of Technology Dresden, Medical Faculty Carl-Gustav Carus, under specific pathogen-free conditions. In all experiments, mice were used at the age of 8–16 weeks. Littermates were used as controls. *Kit^{W-sh/W-sh}* mice were used at the age of 12 weeks to ensure MC deficiency; WT C57BL/6 mice were used as controls. All procedures were in accordance with institutional guidelines on animal welfare and were approved by the Landesdirektion Dresden.

Single-Cell Sorting and Single-Target Amplification of the *Il10* Locus

Single MCs ($\text{Fc}\epsilon\text{RI}^+\text{CD117}^+$), macrophages (F4/80^+), and B cells (CD19^+) from peritoneal lavage as well as MCs ($\text{CD45}^+\text{Fc}\epsilon\text{RI}^+\text{CD117}^+$) and T cells ($\text{CD45}^+\text{CD3}^+$) from ear skin cell suspensions were sorted into thin-walled PCR tubes containing PCR buffer (one cell per tube) with the single-cell deflection module of an FACS Aria II instrument (BD Biosciences). The *Il10* locus of the sorted cells was amplified in a two-round nested PCR (see Supplemental Experimental Procedures). PCR products were verified by sequencing.

Intravital 2-Photon Microscopy of MCs and Vessels in Ear Skin

Mice were prepared for intravital microscopy as previously described (Köhler et al., 2009). In brief, animals were subjected to intubation narcosis with a mixture of isoflurane (1.2%) and oxygen with a mechanical ventilator (Mini-Vent, Hugo-Sachs Elektronik, March-Hugstetten, Germany). Two-photon intravital microscopy was performed with a Zeiss LSM-710 microscope with simultaneous detection via three external non-descanned-detectors (NDDs). Illumination was performed at 950 nm with a MaiTai TiSa-laser (10%–12% laser power, at 1400mW maximum power) via a 20× water-dipping lens with 1.0 NA. EYFP-expressing MCs were detected in the 500–550 nm emission window, blood vessels, and vessel leakage by i.v. injection of 20 μl Qtracker 655 nontargeted Qdots (Invitrogen) in 130 μl PBS (detection 640–760 nm). Skin tissue structure was visualized by its second harmonic generation (SHG) signal (<485 nm). Image series were recorded every 60 s for 15–30 min at a depth of 84–88 μm with 4 μm Z spacing. Single images were recorded as Z stacks encompassing the entire epidermis and dermis (up to a depth of 100–120 μm) and analyzed as extended focus images or surface rendered z-stacks. Raw data were reconstructed using Volocity (Perkin Elmer/Improvision).

Induced MC Depletion

Six- to eight-week-old *Mcpt5-Cre⁺iDTR⁺* and *Cre⁻* littermates received four successive i.p. injections of 25 ng DT/g bodyweight in weekly intervals. Mice were used in experiments 1 week after the last i.p. DT injection. For CHS experiments, mice were treated systemically (4× 25 ng DT/g bodyweight, i.p.) and locally by two successive subcutaneous injections of 5 ng/g DT at the base of the ear pinna 6 days and 2 days before allergen challenge onto the ear.

CHS

Mice were sensitized to various haptens on the shaved back and challenged on the ear. Vehicle controls were sensitized but challenged with the respective

solvent only. Ear thickness was measured before and at several time points after challenge with an engineer's micrometer (Mitutoyo). Ear swelling was determined as the percent increase compared to prechallenge ear thickness. For DNFB, mice were sensitized with 100 μ l 0.5% (w/v) in acetone/olive oil (4:1) and challenged 6 days later with 20 μ l 0.2% (10 μ l each side of the ear). For FITC, mice were sensitized with 100 μ l 1% (w/v) in acetone/dibutylphthalate (1:1) and challenged 6 days later with 20 μ l 0.5% (10 μ l each side of the ear). For urushiol, mice were sensitized with 30 μ l 20mg/ml in 100% acetone and challenged 5 days later with 20 μ l 5 mg/ml (10 μ l each side of the ear). For TNBC, mice were sensitized with 100 μ l 7% (w/v) in acetone/olive oil (4:1) and challenged 6 days later with 20 μ l 1% (10 μ l each side of the ear). For oxazolone, mice were sensitized with 25 μ l 100 mg/ml in acetone/olive oil (4:1) and challenged 5 days later with 20 μ l 10 mg/ml (10 μ l each side of the ear), as described in the study of Grimbaldston et al. (2007). Pyrilamine (10 μ g/g bodyweight) and cromolyn (25 μ g/g bodyweight) were injected i.v. 1 hr before challenge. Anti-Gr1 (10 μ g/g bodyweight) or isotype control antibody (10 μ g/g bodyweight) was injected i.p. 24 hr before challenge.

Analysis of LN Hypertrophy and DC Migration

Mice were sensitized with 0.5% DNFB on the shaved back. Seventy-two hours later, both inguinal LNs were isolated and CD45⁺ cells, CD4⁺ and CD8⁺ T cells, CD19⁺ B cells, and CD11c⁺MHCII^{hi} DCs were quantified by FACS. For evaluation of the DC migration from skin to draining LNs, mice were sensitized with FITC or vehicle only. Numbers of CD45⁺ cells, CD11c⁺ MHCII^{hi} DCs, and FITC⁺CD11c⁺MHCII^{hi} DCs from inguinal LNs were quantified by FACS 48 hr later.

Statistical Analysis

Data are shown as means $\pm$ SD. Statistical analysis was performed with the Student's t test. Significance levels are indicated in each figure.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table, seven figures, six movies, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.immuni.2011.03.028.

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Supplemental Information

Mast Cells Are Key Promoters of Contact Allergy

that Mediate the Adjuvant Effects of Haptens

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Figure legends

Table S1. Determination of specificity and efficiency of Cre-mediated inactivation of the *I110* gene in MCs. Results of the single target amplification of the loxP-flanked *I110* locus from FACS-sorted single MCs and non-MCs from the peritoneal cavity (A) or skin (B). The PCR tubes containing one cell each as well as negative control tubes were subjected to two rounds of amplification as described in the supplementary methods section. Table S1 is related to Figure 2.

Figure S1. Mast cell specificity and efficiency of Cre-mediated recombination in *Mcpt5-Cre* mice. (A) The *Mcpt5-Cre* transgene was bred onto the Cre excision reporter line *ROSA26 Stop^{flox} EYFP (R26Y)*. EYFP expression was analyzed in cell suspensions generated by proteinase digest of ear tissues of *Mcpt5-Cre⁺ R26Y⁺* mice or their *Cre⁻ R26Y⁺* littermate controls. MCs were gated as *CD45⁺ CD117⁺ FcεRIα⁺* cells and their EYFP fluorescence was displayed in histogram plots (upper panels). EYFP⁺ cells were gated and analyzed for CD117 and FcεRIα expression (lower panels, results are representative of 5 animals). (B) Peripheral blood NK cells were gated as *CD49b⁺ NK1.1⁺* cells and their EYFP fluorescence was displayed in histogram plots. % EYFP⁺ or EYFP⁻ cells was presented as mean ±SD, n=5.

Figure S2. No significant changes in the numbers of other hematopoietic cell types in mast cell-depleted mice. MCs were depleted in *Mcpt5-Cre⁺iDTR⁺* mice by four i.p. injections of DT at weekly intervals. Various hematopoietic cell populations in back skin, spleen, bone marrow and blood were analyzed by flow cytometry using the markers indicated. Data represent mean $\pm$ SD of relative cell numbers (% of CD45⁺ cells) in DT injected *Mcpt5Cre⁺iDTR⁺* mice (n=5) compared to DT-injected *Cre⁻* controls (n=5).

Figure S3. Mast cell populations do not recover significantly within three weeks after depletion under steady state or within 8 days after DNFB challenge. MCs were depleted in *Mcpt5-Cre⁺iDTR⁺* mice by four i.p. injections of DT and MC numbers in back skin (A) and peritoneum (B) (CD117⁺FcεRIα⁺ cells) were determined by histology (Giemsa staining) or flow cytometry, respectively, at the time points indicated. (C) *Mcpt5-Cre⁺iDTR⁺* mice and *Cre⁻* littermates controls were treated with four i.p. and two s.c. injections of DT at the base of the ear pinnae to deplete ear skin MCs efficiently. MC numbers were counted in Giemsa-stained sections of ear skin before (non-treated) as well as 24h or 8 days after DNFB challenge, (mean $\pm$ SD, 5 mice per group).

Figure S4. Efficient DT-induced depletion of connective tissue type but not mucosal mast cells in the intestine of *Mcpt5-Cre⁺iDTR⁺* mice. MCs were depleted in *Mcpt5-Cre⁺iDTR⁺* mice by i.p. injections of DT. Numbers of (intraepithelial) mucosal MCs and (subepithelial) connective tissue type MCs were quantified histologically in chloroacetate esterase-stained sections of the stomach.

Figure S5. Immediate response to various sensitizing haptens results in an early transient ear swelling. (A) Ear swelling response to DNFB in sensitized (black square) or non-sensitized (grey square) WT mice (n=5), mean $\pm$ SD. (B) Ear swelling response to urushiol, oxazolone, TNCB and FITC (black circle) or the respective vehicle control (grey circle) as indicated in Experimental Procedures in WT mice (n=5 each group); mean $\pm$ SD.

Figure S6. Quantification of mast cell-degranulation on histological sections of allergen-treated ears.

Ears of non-sensitized mice were treated with the challenge dose of DNFB or FITC. Mice were sacrificed 30 minutes or 1 hour later, ears were fixed in formaldehyde and degranulated MCs were counted on Giemsa-stained paraffin sections. Ear tissue obtained 30 minutes after local injection of the degranulation-inducing agent c48/80 served as a positive control. In the direct vicinity of the injection site, most MCs were significantly degranulated, with MCs appearing as diffuse accumulations of metachromatic granules without distinguishable cell boundaries (arbitrarily designated as “extensively degranulated”). MCs with clearly distinguishable cell boundary but surrounded by numerous granules were classified as “moderately degranulated”. In untreated negative control or allergen-treated ears, the total number of MCs counted in the entire ear was set to 100% and relative frequencies of resting (white bars), moderately degranulated (grey bars) or extensively degranulated MCs (black bars) were determined. n=3 per group, mean $\pm$ SD.

Figure S7. Position of the primers for single target amplification of the loxP-flanked or inactivated (“deleted”) *Il10* locus. First round primers in black, second round primers in grey. The FP2/MP2 product representing the loxP-flanked situation is 323 bp. The FP2/RP2 product representing the deleted situation is 279 bp, the FP2/RP2 product

representing the loxP-flanked situation is 2810 bp (not amplified in most instances).
Not drawn to scale.

Supplemental Experimental Procedures

Chemicals and reagents.

DNFB (1-fluoro-2,4-dinitrobenzene), TNCB (2-chloro-1,3,5-trinitrobenzene), FITC (fluorescein-isothiocyanate), oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one), pyrilamine, cromolyn, diphtheria toxin, and hyaluronidase were obtained from Sigma-Aldrich. Urushiol (3-pentadecylcatechol) was purchased at LGC Standards, Wesel, Germany.

The following monoclonal antibodies directed against mouse antigens were obtained from eBiosciences: CD117 (clone 2B8), Fc ϵ RI α (MAR-1), CD45 (30-F11), CD11c (N418), F4/80 (BM8), CD11b (M1/70), CD4 (RM4-5), CD8a (53-6.7), CD19 (eBio1D3), CD3 (145-2C11), CD49b (DX5), NK1.1 (PK136), I-A b (AF6-120.1), Gr-1 (RB6-8C5). For neutrophil depletion, an anti-Gr1 mAb (RB6-8C5, “functional grade” purification) and an IgG2 α k isotype control from eBioscience was used. 7-AAD viability stain was obtained from eBioscience. Liberase TM and DNase I were obtained from Roche.

Histology and quantification of mast cells.

Biopsies of abdominal skin, back skin or ear pinna were fixed in 4% (v/v) buffered formalin and were embedded in paraffin (ensuring a cross-sectional orientation). 5 μ m sections were metachromatically stained (Giemsa) for the detection of MCs. MCs were counted in five consecutive microscopic fields at a final magnification of 200x.

The number of MCs in MC-depleted *Mcpt5-Cre⁺ iDTR⁺* mice or MC-deficient *Mcpt5-Cre⁺ RDTA⁺* mice was compared to Cre- littermate controls. The number of MCs in MC-deficient *Kit^{W/W-v}* mice was compared to *Kit^{+/+}* WBB6F1 littermate controls. The number of MCs in MC-deficient *Kit^{W-sh/W-sh}* mice (C57BL/6 background) was compared to age- and sex-matched C57BL/6 WT control mice.

Preparation of cell suspensions and FACS analysis.

For preparation of skin cell suspensions, samples of ear tissue were cut into small pieces and digested in 1ml DMEM containing 20mM Hepes, 0.025 mg/ml Liberase TM, 396 U/ml DNase I, and 0.5 mg/ml Hyaluronidase at 37°C, 1400rpm for 1-2h. Samples were passed through a 40µm sieve and the cell suspension was washed twice with PBS.

Cells isolated from skin, spleen, lymph nodes or blood were resuspended in PBS/2% BSA. $2-5 \times 10^5$ cells were stained with monoclonal antibodies in 100µl staining solution for 30' at 4°C. Cell suspensions were washed twice and were resuspended in 200µl PBS/2% BSA. To determine cell viability, 2µl 7-AAD viability stain was added. Analysis was done using the Miltenyi MacsQuant flow cytometer with MacsQuant™ Analysis Software.

Single cell PCR.

The single cells were sorted into thin-walled PCR tubes containing 20 µl 1x Dream Taq PCR buffer (Fermentas) and stored at -80°C. After thawing, proteinase K (Fermentas) was added (0.25 mg/ml final concentration) and the cells were incubated at 50°C for 2 h followed by inactivation of the enzyme by heating to 95°C for 10 min. Single target PCR was performed in two rounds of amplification using nested primers in the second round as illustrated in Figure S7. 29.5 µl of a first round reaction mix

were added containing all reagents except for the Taq polymerase. Finally, the 50µl reaction was 1x Dream Taq PCR buffer (2 mM MgCl₂), 125 µM each of dATP, dTTP, dGTP and dCTP, and 16.6 nM each first round primer (FP1 5'-ATT-ACG-GGA-AGA-TAT-AAG-AGG-TGC-TG-3', MP1 5'-GAC-CTG-TAC-TTA-CTG-CAC-GGT-GA-3', RP1 5'-AAG-TTA-GAC-CCA-GGC-CCG-GT-3'). An initial cycle of 95°C for 3 minutes, a pause at 80°C, during which 0.5 µl of Dream Taq (5 U/µl, Fermentas) were added, and 72°C for 90 seconds was followed by 35 cycles of 95°C for 30 seconds, 60°C for 45 seconds and 72°C for 2 minutes. Finally the reaction was incubated at 72°C for 5 minutes.

1 µl of the first round reaction was added to two separate second round reactions (primer FP2 5'-CGG-GAA-GAT-ATA-AGA-GGT-GCT-GC-3' combined with primer RP2 (5'-GTT-AGA-CCC-AGG-CCC-GGT-CT-3') or primer MP2 (5'-GTA-CTT-ACT-GCA-CGG-TGA-TAG-CC-3')). Second round PCR was performed in a volume of 35 µl with 1x Dream Taq buffer (2 mM MgCl₂), 200 µM each of dATP, dTTP, dGTP and dCTP, 200 nM each primer. 0.35 µl Dream Taq polymerase (5 U/µl) was added. An initial cycle of 95°C for 3 minutes, 65°C for 90 seconds and 72°C for 2 minutes was followed by 44 cycles of 95°C for 30 seconds, 65°C for 45 seconds and 72°C for 2 minutes, and a final 5 minute incubation at 72°C.

A) peritoneal cavity

Cell type	Products representing deleted locus	Products representing loxP-flanked locus	No product	Total number of cells analyzed
<i>Mcpt5-Cre⁺ II10^{fl/fl}</i>				
Mast cells	11	-	4	15
B cells	-	7	10	17
Macrophages	-	7	10	17
<i>Cre⁺ II10^{fl/fl}</i>				
Mast cells	-	9	4	13
B cells	-	5	11	16
Macrophages	-	9	7	16
Sorting control				
	-	-	6	6
Water control				
	-	-	7	7

B) Skin cell suspension

Cell type	Products representing deleted locus	Products representing loxP-flanked locus	No product	Total number of cells analyzed
<i>Mcpt5-Cre⁺ II10^{fl/fl}</i>				
Mast cells	7	-	14	21
T cells	-	3	13	16
<i>Cre⁺ II10^{fl/fl}</i>				
Mast cells	-	3	17	20
T cells	-	5	7	12
Sorting control				
	-	-	5	5
Water control				
	-	-	5	5

Table S1.

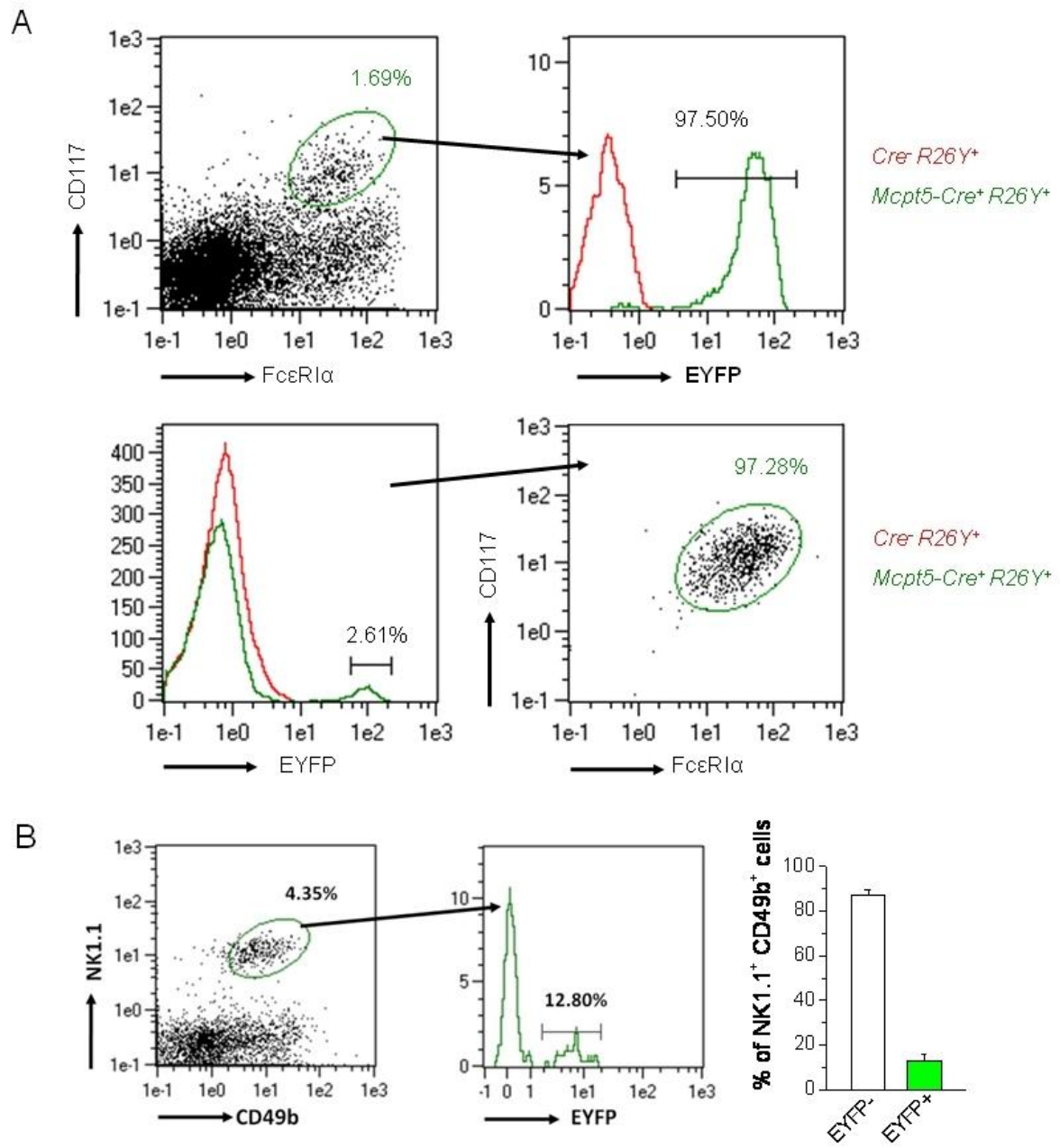


Figure S1

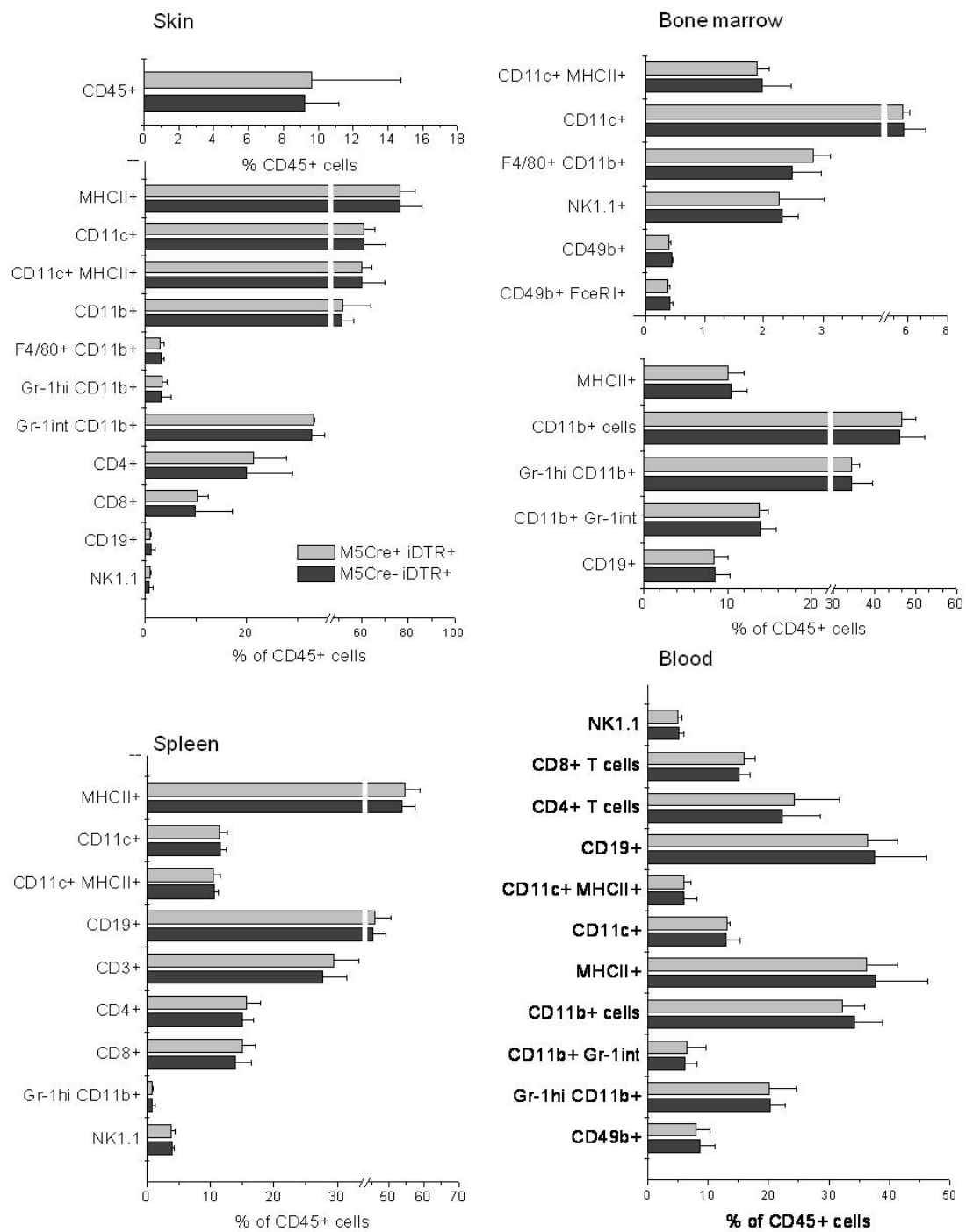


Figure S2

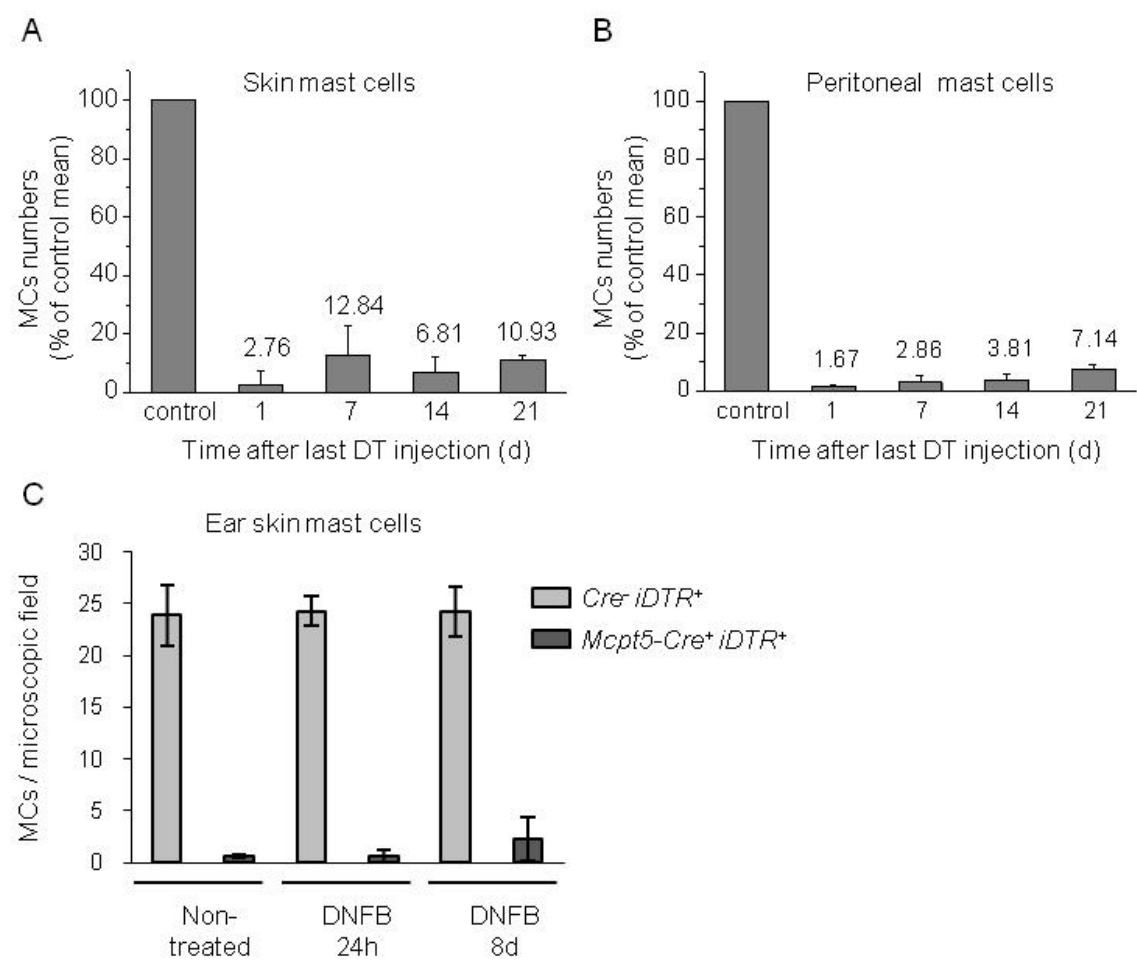


Figure S3

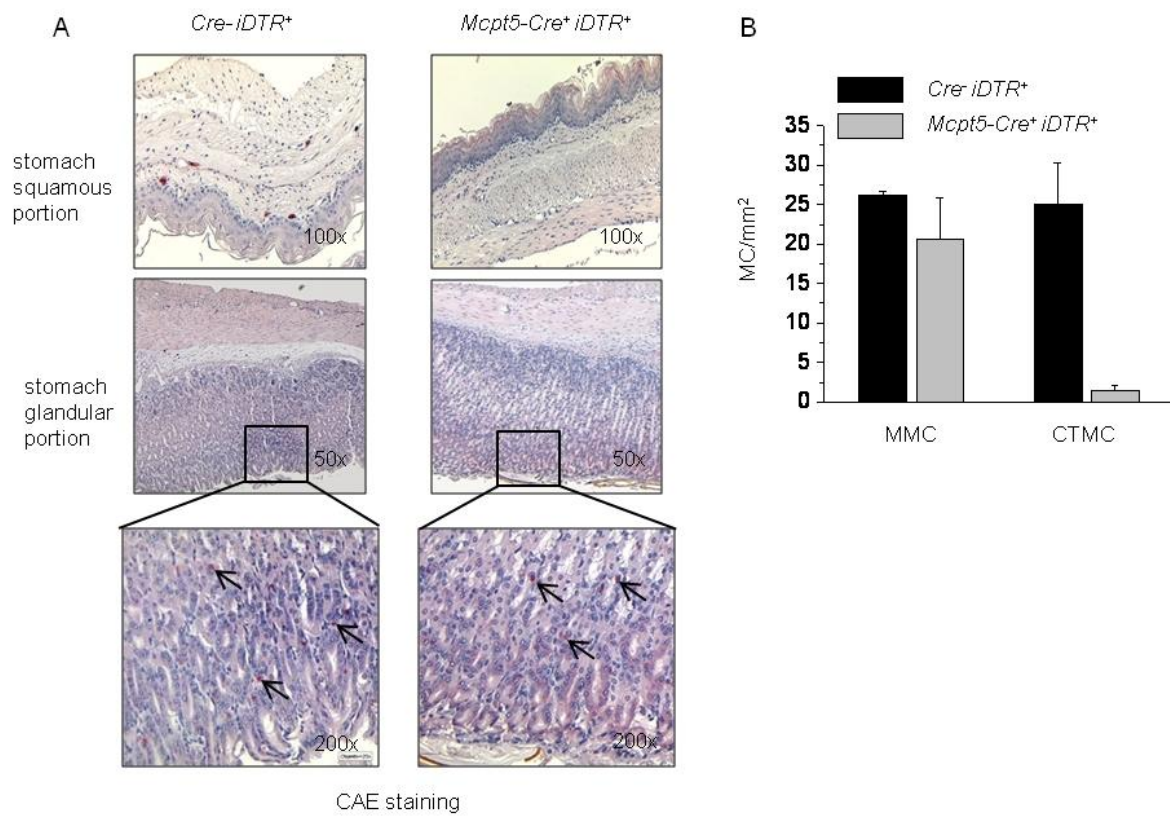


Figure S4

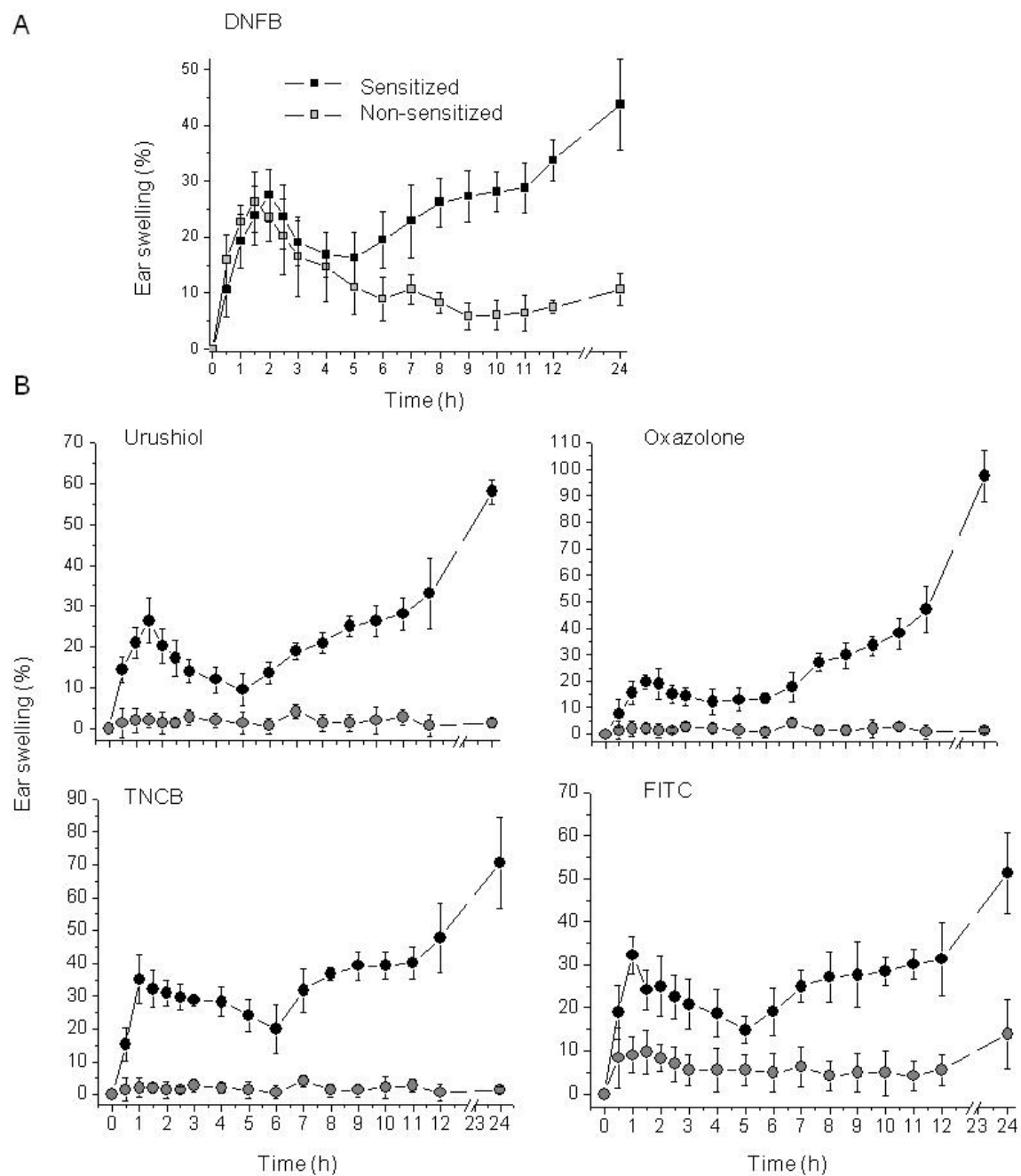


Figure S5

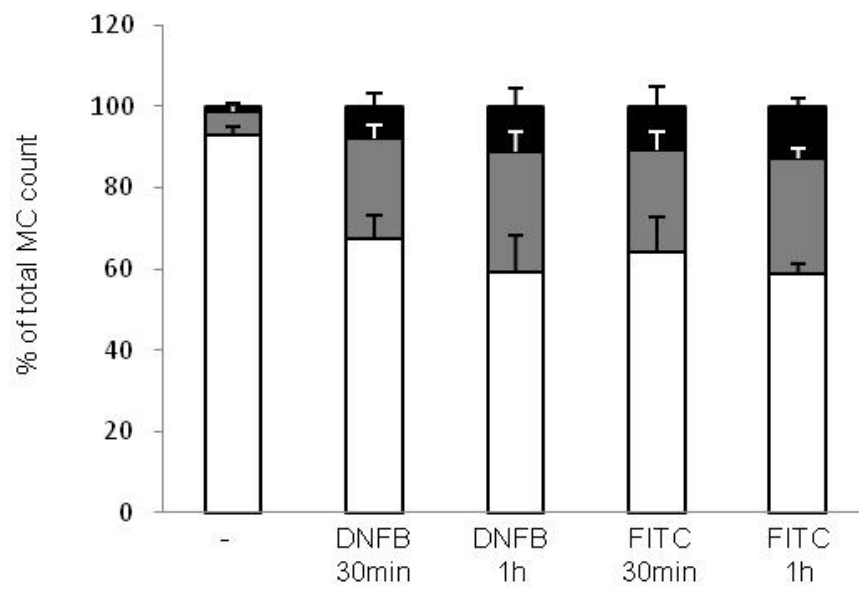
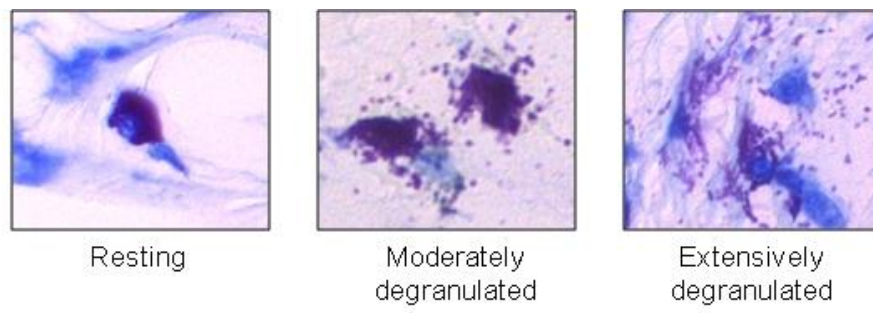


Figure S6

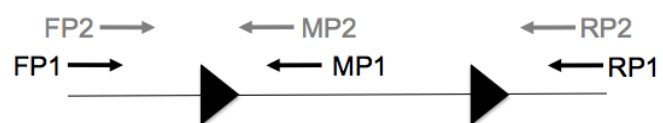


Figure S7.