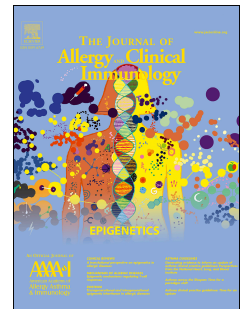


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Mast cells initiate the vascular response to contact allergens by sensing cell stress

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of interest.

Abbreviations

CHS: contact hypersensitivity; DC: dendritic cell; DNFB: 2,4-Dinitrofluorobenzene; MC: mast cell

Key words

Mast cells, contact allergy, skin inflammation, neutrophil granulocytes, cell stress, ATP, IL-33

Capsule Summary

Mast cells (MCs) contact hypersensitivity responses, but the mode of MC activation is not yet understood. Hoppe et al. show, that MCs trigger an immediate edema formation and neutrophil infiltration by sensing cell stress concomitantly via the ATP/P2X7 and IL-33/ST2 pathway.

To the editor

While Mast cells (MCs) are well known as key effector cells of IgE-mediated type I allergy, there is growing evidence that MCs are also essential players in innate and adaptive immunity, and in many inflammatory or autoimmune diseases ¹. IgE crosslinking, but also IgE-independent mechanisms of MC activation, result in degranulation of MC secretory granules containing preformed pro-inflammatory mediators including histamine, cytokines and proteases.

By studying hapten-induced contact hypersensitivity (CHS) responses in novel transgenic mouse lines of MC deficiency or conditional gene knockout, we have previously demonstrated that MCs are of critical impact in the pathomechanism of allergic contact dermatitis. More specifically, we have shown that MCs enhance the migration of dendritic cells (DCs) to draining lymph nodes upon hapten sensitization, thereby promoting effector T cell expansion ^{2,3}. Upon challenge (second contact with the same hapten), MCs are required for efficient skin homing of T cells and thereby facilitate the T cell-driven skin inflammation. In our previous study, longitudinal intravital imaging of the immune events following hapten contact had surprisingly revealed an immediate MC-triggered vascular response, happening irrespectively of previous sensitization and being crucial for subsequent T cell-driven skin inflammation ². However, this IgE-independent mode of MC activation during allergic contact dermatitis is not yet clarified.

In this study, we demonstrate that epicutaneous DNFB administration caused a pronounced vasodilatation of dermal blood vessels within the very first minutes, which was followed by a massive leakage of the intravascular dye due to increased vessel permeability (Figure 1A). The edema formation could be measured as an early ear swelling response peaking at 2h post DNFB (Figure1B) and was almost completely abolished in MC-depleted mice (Figure 1A, ²). In sensitized mice, the early ear swelling response was followed by a second T cell-driven edema at 24h after DNFB. However, the

amplitude and kinetics of the early ear swelling was similar in non-sensitized mice (in contrast to the 24h ear swelling) indicating its innate nature. The hapten-induced innate response further comprised a MC-driven neutrophil infiltration. The early ear swelling response was impeded by treatment with the MC silencer cromolyn or with pyrilamine, a histamine receptor antagonist, prior to DNFB administration, suggesting a mechanism dependent on MC degranulation and histamine release (Figure 1C). In contrast, the adaptive skin inflammation (at 24h post DNFB) was significantly reduced in cromolyn-treated mice compared to pyrilamine treatment indicating additional granule-embedded MC-mediators beside histamine (Figure 1C). Given the fast MC degranulation within only few minutes post DNFB, we questioned the underlying mode of MC activation. Previous reports demonstrated that the absence of TLR-signaling in DCs prevented DC-mediated sensitization, effector T cell expansion, and subsequent CHS responses⁴. Therefore, we studied the innate CHS response to DNFB in *TLR2/4*^{-/-} mice and found the MC-driven early vascular response to be not affected by concomitant absence of TLR2 and TLR4, while, confirming previous findings, the adaptive skin inflammation at 24h was markedly reduced (Figure S1).

Previous reports demonstrated that epicutaneous hapten treatment results in a rapid release of extracellular ATP by keratinocytes that is subsequently sensed by DCs via the purinergic receptor P2X7⁵. In addition, IL-33, an alarmin of the IL-1 family being released upon cell stress or necrotic cell death, has been shown to impact on DNFB-induced CHS⁶.

Questioning whether danger signaling might be involved in the rapid hapten-induced MC activation, we found a prominent surface expression of the ATP receptor P2X7 as well as the IL-33 receptor ST2 on a fraction of about 40% and 65%, respectively, of mouse ear skin MCs under healthy conditions (Figure 1D). Given that expression, we assessed whether antagonizing the P2X7 ligand binding or neutralizing IL-33 affects the amplitude of the MC-mediated early ear swelling response and neutrophil infiltration. We could show that inhibiting the ATP/P2X7 signaling axis using the antagonist KN62 markedly reduced the innate ear swelling while not significantly affecting the adaptive response at 24h and neutrophil infiltration (Figure 1E/F). In contrast, neutralizing IL33 signaling by either an anti-IL-33 antibody or a soluble IL-33 receptor dramatically reduced the innate ear swelling but also significantly prevented the adaptive inflammation and neutrophil recruitment to the challenged skin (Figure 1G/H). This data could be confirmed using *P2X7*^{-/-} mice showing a reduced innate and adaptive edema formation upon hapten treatment (Figure 1I). To discern P2X7 function in MCs, we employed *Mcpt5-Cre P2X7*^{FL/FL} mice, which lack P2X7, specifically in connective tissue type MCs. Importantly, *Mcpt5-Cre*⁺*P2X7*^{FL/FL} (referred to as MC^{ΔP2X7}) showed a marked reduction of innate and adaptive ear swelling compared to *Cre*⁻*P2X7*^{FL/FL} littermates, but neutrophil infiltration to challenged ear skin was not affected (Figure 1J/K). These findings suggest that MCs sense

extracellular ATP released upon DNFB-induced cell stress or damage by secreting the vasoactive compound histamine. Since, in CHS, MCs trigger both the immediate vascular response, as well as neutrophil infiltration², we speculated that IL-33 signaling might play an additional role in cell stress-related MC activation. Hence, we studied CHS in $ST2^{-/-}$ mice lacking the IL-33 receptor, and detected a diminished innate and adaptive ear swelling (Figure 1L). Specific IL-33 sensing by MCs via ST2 was proven using MC-deficient $Mcpt5-Cre^{+}RDTA$ mice which were reconstituted, locally in the ear skin, with *in vitro* expanded peritoneal MCs (PMCs) from either $ST2^{-/-}$ mice (referred to as $\Delta MC+MC^{\Delta ST2}$) or wt mice ($\Delta MC+MC^{wt}$) (Figure 1M). $\Delta MC+MC^{\Delta ST2}$ mice showed decreased innate and adaptive ear swelling post DNFB, as well as a significant reduction in neutrophil numbers infiltrating the challenged ear skin, compared to $\Delta MC+MC^{wt}$ (Figure 1N).

Translation of extracellular ATP sensing by MCs into histamine-driven early vascular responses was confirmed by intradermal injection of ATP into $P2X7^{-/-}$ mice (Figure 2A/B), $MC^{\Delta P2X7}$ (Figure 2C/D) and MC-deficient $Mcpt5-Cre$ RDTA (ΔMC) mice (Figure 2E/F). I.d. injection of ATP into mouse ear pinnae resulted in a short-term, self-resolving ear swelling, peaking at 1h post injection, while a late ear swelling and neutrophil infiltration at 24h was not significantly induced. Importantly, the early ear swelling response was significantly reduced in $MC^{\Delta P2X7}$ mice, but completely abolished in ΔMC mice, suggesting additional ATP sensing via other receptors (Figure 2C/E). In contrast, i.d. injection of IL-33 resulted not only in a prominent early ear swelling, peaking at 2h post injection, but also in pronounced skin inflammation at 24h, accompanied by massive neutrophil infiltration (Figure 2G/H). All these events were significantly reduced in $ST2^{-/-}$ mice and hence, directly induced by IL-33/ST2 binding. Confirming the *in vivo* findings, we found that MC stimulation with ATP *in vitro* resulted in prominent degranulation and histamine release but not in TNF production (Figure 2I-K) while in contrast, IL-33 has been demonstrated to induce TNF production *in vitro* and MC degranulation *in vivo* but not *in vitro*⁷.

Our data indicate that IL-33 signaling by MCs and amplification of stress responses by MC degranulation may be worth to be studied in more detail in the context of human inflammatory skin diseases. The observed mechanisms should impact on therapeutic decisions in cases of contact sensitivity which may be difficult to manage. Here, for instance a local treatment with antihistamines and MC stabilizers as cromolyn glycates, or even local neutralization of IL-3 or TNF might be a conceivable strategy. Indeed, the IL-33/ST2 signaling axis has been suggested to contribute to the pathomechanism of atopic dermatitis and currently, first clinical trials targeting IL-33 as a novel therapeutic strategy yield promising results^{8,9}.

Collectively, we herein demonstrate that, upon hapten encounter, MCs rapidly initiate innate vascular responses including vasodilatation, vessel permeabilization and neutrophil influx by prompt degranulation. This MC activation in context of hapten-induced CHS is a consequence of sensing cell stress or damage by concomitant signaling via the IL33/ST2 and ATP/P2X7 axis. While ATP sensing by MCs is sufficient for inducing the histamine triggered edema formation, additional IL33 sensing is required for efficient induction of neutrophil infiltration.

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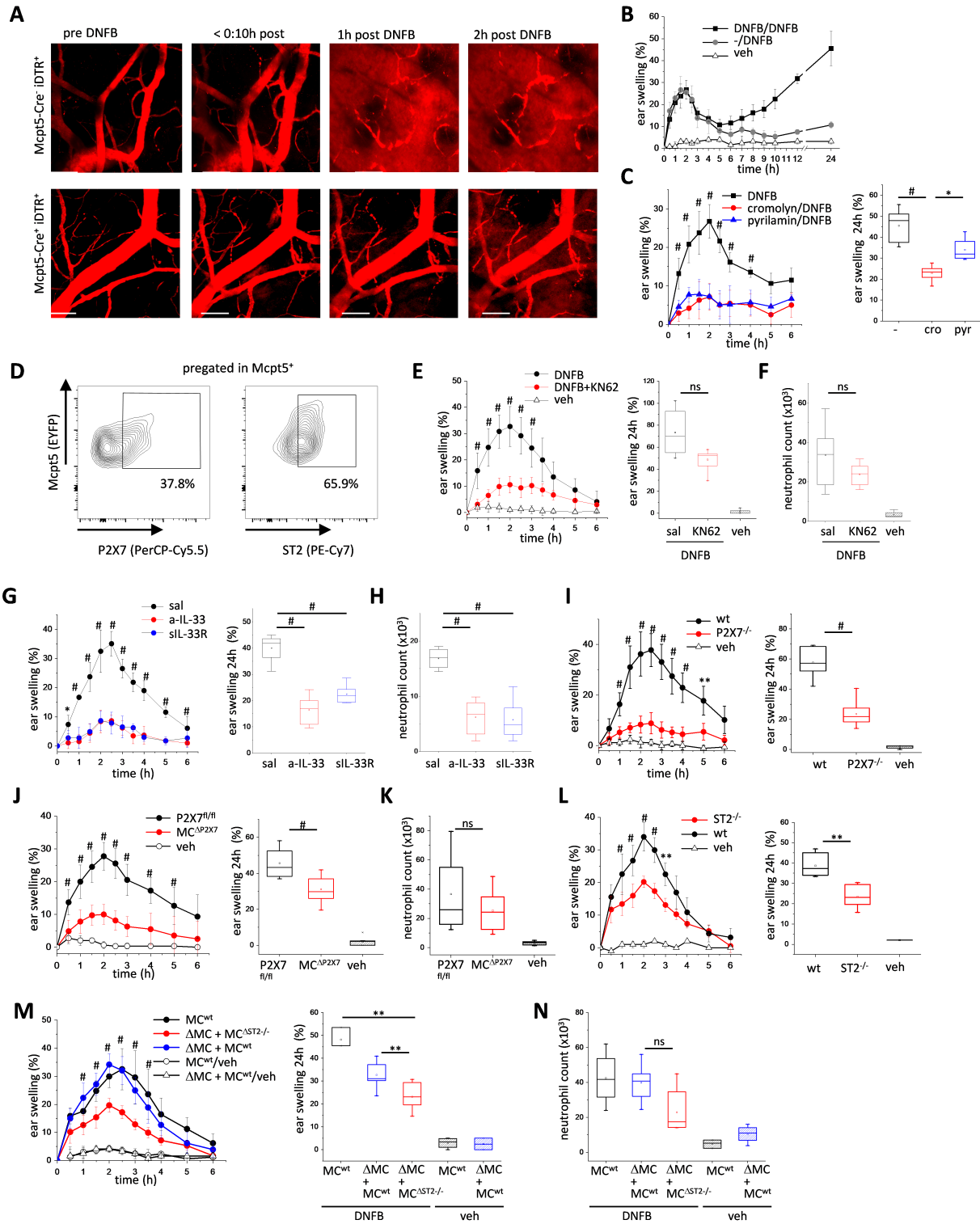
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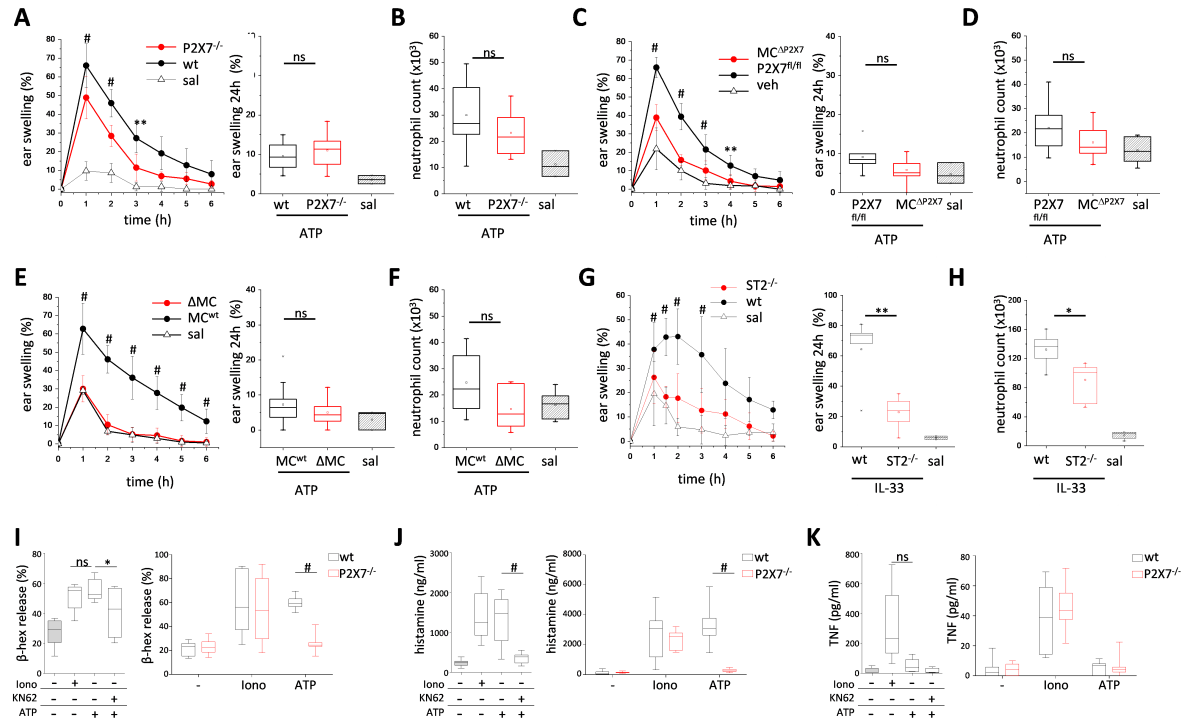
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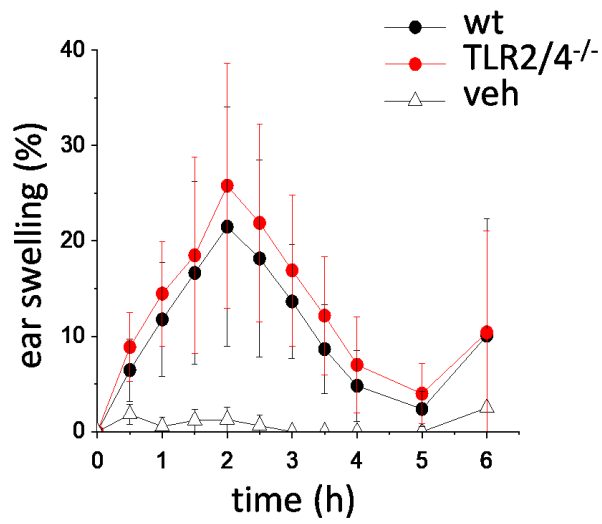
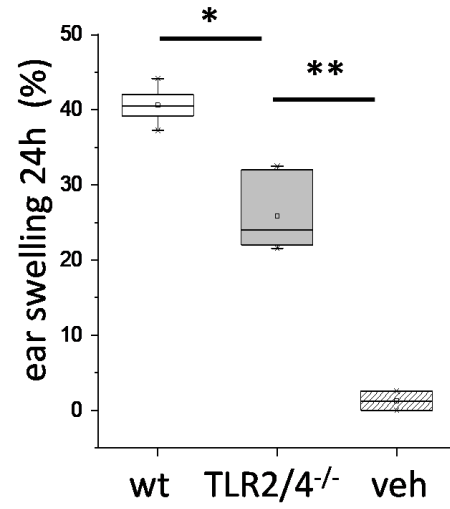
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Figure 1. Mast cells trigger innate vascular responses to hapten challenge via P2X7 and ST2 signaling. (A) Longitudinal intravital 2-photon microscopy of Mcpt5-Cre⁺iDTR mice and Cre⁻ littermates before and after DNFB challenge. (B) Ear swelling post DNFB in sensitized (DNFB/DNFB) and non-sensitized (-/DNFB) mice and vehicle controls, (n=6, veh n=4). (C) Ear swelling post DNFB in wt (black) and wt pretreated with cromolyn (red) or pyrilamine (blue), n=6, # p<0.001 vs. untreated. (D) P2X7 and ST2 receptor expression by ear skin MCs. (E-N) Ear swelling was measured at 0-6h and 24h, and neutrophil infiltration was quantified at 24h post DNFB compared to vehicle control (veh). (E/F) Wt (black) and wt pretreated with KN62 (red) (n=12; veh n=8). (G/H) Wt pretreated with IL-33 blocking antibody (a-IL-33, red, n=6), soluble IL-33 receptor (sIL-33R, blue, n=6), or saline (sal, black, n=4). *p<0.05, #p<0.001 vs. sal. (I) Wt (black) and P2X7^{-/-} mice (red) (n=12, veh n=8). (J/K) Mcpt5-Cre⁺P2X7^{FL/FL} (MC^{ΔP2X7}) mice (red) compared to Cre⁻ littermates (n=16, veh n=8). (L) wt (black) and ST2^{-/-} mice (red) (n=8, veh n=3). (M/N) Mcpt5-Cre⁺R-DTA mice reconstituted with wt PMCs (ΔMC+MC^{wt}, blue) or ST2^{-/-} PMCs (ΔMC+MC^{ΔST2}, red) compared to Cre⁻RDTA littermates (MC^{wt}, black), and vehicle treated Cre⁻ controls (MC^{wt}/veh) and Mcpt5-Cre⁺RDTA reconstituted with wt PMCs (ΔMC+MC^{wt}). (n=8, veh n=4); *p<0.05, #p<0.001 blue vs. red.

Figure 2. Mast cells respond to cell-stress mediators ATP and IL-33 in vivo and in vitro. Ear swelling was measured at 0-6h and 24h after i.d. injection of ATP or IL-33 as compared to PBS-treated controls (n=4-5) and neutrophil infiltration was measured 24h post injection by flow cytometry. (A) Ear swelling and (B) neutrophil influx in wt and P2X7^{-/-} mice upon ATP i.d. (n=12 per group). (C) Ear swelling and (D) neutrophil influx in Mcpt5-Cre⁺P2X7^{FL/FL} (MC^{ΔP2X7}) and Cre⁻ littermates (P2X7^{FL/FL}) (n=10 per group) upon ATP i.d. (E) Ear swelling and (F) neutrophil infiltration in MC-deficient Mcpt5-Cre⁺RDTA mice (ΔMC) and Cre⁻ littermates (MC^{wt}) (n=12 per group) upon ATP i.d. (G) Ear swelling and (H) neutrophil influx upon i.d. injection of IL-33 in wt and ST2^{-/-} mice (n=12 per group). (I/J/K) The release of β-hexosaminidase (I), histamine (J) and TNF (K) upon stimulation with ATP in absence or presence of KN62 and compared to A23187 calcium-ionophore treatment as positive control (iono) by *in vitro* expanded peritoneal MCs (PMCs) from wt mice (left panels, n=11) or P2X7^{-/-} mice (right panels, n=11 in (I), n=7 in (J) and (K)).





A**B**

Mast cells initiate the vascular response to contact allergens by sensing cell stress

Anja Hoppe, MSc¹, Konstantinos Katsoulis-Dimitriou, PhD², Hanna Edler, MSc², Jan Dudeck, MSc^{2,3}, Sebastian Drube, PhD⁴ and Anne Dudeck, PhD^{2,3}

SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Mice. All animal experiments were conducted in accordance with German guidelines and were approved by the relevant local authorities in Magdeburg and Dresden. Mcpt5-Cre mice ¹ were provided by A. Roers (Dresden, Germany), R-DTA mice ² by D. Voehringer (Erlangen, Germany), TLR2/4^{-/-} mice ³ were kindly provided by S. Martin (Freiburg, Germany) and P2X7^{-/-} and P2X7^{FL/FL} mice ⁴ by M. Idzko (Freiburg, Germany). C57BL/6 wt mice were purchased from Janvier. In all experiments, mice were housed under specific pathogen free conditions and were used at the age of 8–16 weeks. Littermates were used as controls.

Reagents. DNFB, ATP, Hyaluronidase, KN-62, A23187, IL-33 and PI were purchased from Sigma Aldrich. DNase and Liberase was obtained from Roche. The goat polyclonal IL-33 blocking antibody (AF3626) was purchased by R&D Systems⁵. Monoclonal antibodies for flow cytometry analysis directed against the following marker proteins were purchased from eBioscience: CD45 (30-F11), F4/80 (Bm8), CD11b (M1/70), CD117 (2B8), and FcεRIα (MAR-1). Antibodies directed against Ly6G, P2X7R (1F11) and IL33R (DIH4) were purchased from BioLegend. Recombinant Mouse ST2/IL-33R Fc Chimera Protein was obtained from R&D Systems.

Intravital 2-photon microscopy of mouse ear skin. Longitudinal and non-invasive intravital 2-photon microscopy of mouse ear skin was performed as previously described ^{6,7}.

CHS. Mice were sensitized with 100 µl 0.5% DNFB (v/v) in acetone/olive oil (4:1) and challenged 6 days later with 20 µl 0.2% DNFB at the ear (10 µl each side of the ear). Vehicle controls were sensitized and challenged with the acetone/olive oil solvent only ⁶. Ear thickness was measured before and at the indicated time points after challenge with an engineer's micrometer (Mitutoyo). Ear swelling was determined as the percent increase compared to pre-challenge ear thickness. 24h after challenge ear skin samples were used for flow cytometry analysis.

Flow cytometry of ear skin biopsies. For analysis of cell infiltration, ear skin samples were cut into small pieces and digested in medium containing 20mM Hepes, 0,025mg/ml Liberase, 396U/ml DNase I and 0,5mg/ml Hyaluronidase for 1h at 37° C and 1400rpm on a thermo mixer. Cell suspensions were

passed through a 40µm sieve, washed twice with PBS/2% BSA and stained in 100µl PBS/2% BSA with the respective antibodies for 30min at 4°C. Neutrophil granulocytes were assessed as CD45⁺Ly6G⁺CD11b⁺F4/80⁺ cells. Infiltrated cells were quantified using Miltenyi MACS Quant or BD fortessa flow cytometer and MACS Quant Analysis software or FlowJo.

Cromolyn and pyrilamine pretreatment. Pyrilamine (10 mg/g bodyweight) and cromolyn (25 mg/g bodyweight) were injected i.v. 1 hr before challenge ⁶.

KN62, anti-IL33 antibody and sIL33R pretreatment. 20µl KN-62 at a final concentration of 178,5µg/ml were injected i.d. into the ear pinna 4h before challenge, control mice received an injection of 20µl saline ⁸. 1µg of anti-IL33 blocking antibody⁵ or soluble IL33 receptor^{9,10} in 20µl saline, was injected i.d. into the ear pinna 1h before challenge, control mice received an injection of 20µl saline only.

ATP and IL-33 injection. 20µl ATP at a final concentration of 826,7µg/ml (1,5µM) were injected i.d. in the ear pinna. 10µl IL-33 (500 ng) was injected i.d. into the ear pinnae. Ear swelling was measured directly after the injection at the indicated time points and FACS analysis were performed at 24h post injection as described above.

MC reconstitution. MC-deficient *Mcpt5-Cre⁺RDTA* mice were locally reconstituted with *in vitro* expanded peritoneal MCs (PMCs) isolated from wt or *ST2^{-/-}* mice by i.d. injection of 1x10⁶ PMCs in 20 µl sterile saline into the ear pinnae as previously proven to result in MC numbers comparable to healthy skin¹¹. Following experiments were conducted at least two weeks after reconstitution.

***In vitro* expansion of peritoneal MCs.** Peritoneal MCs (PMCs) were isolated and cultured as previously described ¹². In brief, peritoneal MCs were isolated by flushing the peritoneal cavity with 5ml ice cold PBS. Cells were pelleted for 5min at 4° C and 1200rpm, resuspended in 5ml RPMI containing 10%FCS, 1%Penicillin/Streptomycin, 1%L-Ala-L-Glu, 1% sodiumpyruvate, supplemented with 10ng/ml IL-3 and 10-30ng/ml SCF and cultured for 8 days at 37°C and 5% CO₂. If required, PMCs were enriched using CD117 MicroBeads (Miltenyi). Purity for reconstitution or following *in vitro* experiments was ≥95% as determined by flow cytometry using antibodies against CD117 and FcεRIα.

***In vitro* ATP stimulation.** PMCs were plated in a 96-Well Plate at a density of 2x10⁵ cells/200µl/well, and stimulated with 500µM ATP in non-conditioned RPMI. Supernatant was collected after 10min and 24h. PMCs stimulated with the calcium ionophore A23187 (500ng/ml) served as a positive control, PMCs cultured in RPMI alone served as negative control.

β-Hexosaminidase assay, histamine and TNF ELISA. Histamine (DRG) and TNF (TNF-α ELISA Max™ Standard from Biolegend) release was quantified using respective ELISAs according to the manufacturer's instructions. MC degranulation was assessed by means of β-Hexosaminidase Assay. 25μl cell supernatant and cell lysate respectively were mixed with 25μL 1mM p-nitrophenyl-N-acetyl β-D-glucosamine in citrate buffer (0,1M; pH4,5) and incubated for 1h at 37°C. The reaction was stopped with 200μl carbonate buffer (0,1M Na₂CO₃; 0,1M NaHCO₃; pH9) and OD was measured at 405nm. Release of β-Hexosaminidase was calculated as percent release of total content (100x amount supernatant/amount supernatant+cell lysate).

Statistical Analysis. Data are shown as mean±SD. Data are pooled from 1 (Fig.1B, D, G, H, L), 2 (Fig. 1 C, E, F, M, N; Fig.2A, B, F, G, H) or 3 (Fig. 1 I, J, K; Fig.2C, D, E, I, J, K; Fig.S1) independent experiments. Statistical analysis was performed with non-parametric one-way ANOVA for all the graphs that contain more than 2 groups and 2-way ANOVA for the ear swelling curves. For the ear swelling curves, unless stated otherwise in the figure legend, the difference depicted is between the red and the black curve. # p <0.001, ** p <0.01 and * p <0.05.

SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

SUPPLEMENTAL FIGURE 1

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Early innate vascular responses are independent of TLR2/4 signaling. C57BL/6 wt mice (n=14) and TLR2/4^{-/-} (n=16) mice were sensitized with DNFB on the shaved back and challenged 6 days later with DNFB on the ear skin. Control mice (veh, n=5) were challenged with the solvent only. The ear swelling was measured (A) at 0-6h and (B) at 24h post DNFB challenge.

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