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Skin Mast Cells Protect Mice against Vaccinia Virus by Triggering Mast Cell Receptor S1PR2 and Releasing Antimicrobial Peptides

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Mast cells (MCs) are well-known effectors of allergic reactions and are considered sentinels in the skin and mucosa. In addition, through their production of cathelicidin, MCs have the capacity to oppose invading pathogens. We therefore hypothesized that MCs could act as sentinels in the skin against viral infections using antimicrobial peptides. In this study, we demonstrate that MCs react to vaccinia virus (VV) and degranulate using a membrane-activated pathway that leads to antimicrobial peptide discharge and virus inactivation. This finding was supported using a mouse model of viral infection. MC-deficient (Kitwsh/−/−) mice were more susceptible to skin VV infection than the wild type animals, whereas Kitwsh/−/− mice reconstituted with MCs in the skin showed a normal response to VV. Using MCs derived from mice deficient in cathelicidin antimicrobial peptide, we showed that antimicrobial peptides are one important antiviral granule component in vivo skin infections. In conclusion, we demonstrate that MC presence protects mice from VV skin infection, MC degranulation is required for protecting mice from VV, neutralizing Ab to the L1 fusion entry protein of VV inhibits degranulation apparently by preventing S1PR2 activation by viral membrane lipids, and antimicrobial peptide release from MC granules is necessary to inactivate VV infectivity. The Journal of Immunology, 2012, 188: 345–357.

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Abbreviations used in this article: MC, mast cell; PtL, multiplicity of infection; PL, propidium iodide; SCF, stem cell factor; SIP, sphingosine-1-phosphate; S1PR2, S1P2 G-coupled receptor; VV, vaccinia virus; WT, wild type.

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lesions or rashes (25, 26). VV infection is a well-established in vivo model for study of skin infection (23, 24) (27–29). We therefore chose this mouse model to study the interaction of skin MCs and VV.

Early reports indicated that VV enters cells through different routes including endocytosis (30, 31) and plasma membrane fusion (32–36). Recently VV has been shown to enter cells both by fusion with the plasma membrane and endocytic vacuoles depending to some extent on the virus strain and cell type (37, 38). The endocytic pathway involves macropinosis (39) or fluid phase uptake (40). In our study, we provide evidence that fusion of the mature virion is required to start the VV–MC interaction and response. The cell-derived lipid membranes of both the mature virion and enveloped virosoma contain many lipids including sphingomyelin (41). Sphingomyelin in the cell membrane can be converted to sphingosine-1-phosphate (SIP1), which can activate the SIP2 G-coupled receptor (SIPR2) in an autocrine manner to stimulate MC degranulation (42–44). We will present data that demonstrate that this pathway is activated upon VV encounter and leads to MC degranulation.

There have been a few reports of MC involvement in viral infections through the initiation of a chemokine-dependent host response (45–50), and of histamine release in response to viral contact (45, 51, 52); however, the direct capacity of MCs to kill VV through antimicrobial peptides has not been reported. In this study, we show that MCs sense, degranulate, and can subsequently kill VV using their antimicrobial peptides. MCs derived from mice deficient in c-kit, which are deficient in c-kit and cathelicidin, we demonstrate that cathelicidin is a critical antiviral granular component in vivo and in vitro. This study provides a novel insight into the role of MCs in antiviral response, the interaction of viruses with MCs, and suggests unexplored therapeutic avenues to resist viral infections.

Materials and Methods

**Mouse**

Mast cell-deficient (C57BL/6-Kit<sup>ε-/ε-</sup>) mice were a donation from Dr. Peter Besmer’s laboratory (Developmental Biology Program, Memorial Sloan-Kettering Cancer Center at Cornell University, New York, NY). The animals were bred at our facility. The Veteran Affairs and Institutional Animal Care and Use Committee (IACUC) approved all animal experiments. These mice have been extensively studied since they were generated (53, 54). Kit<sup>ε-/ε-</sup> mice bearing the W-sash (W<sup>sh</sup>) inversion mutation have MC deficiency but lack anemia and sterility. Adult Kit<sup>ε-/ε-</sup> mice have a profound deficiency in MCs in all tissues examined, but normal levels of major classes of other differentiated lymphoid cells. In adulthood, these mice may develop myeloid and megakaryocyte dysplasia in the spleen (55, 56). In our case, 20–30% mice exhibit splenomegaly. Hematopoietic abnormalities extend to the bone marrow and are reflected by neutrophilia and thrombocytosis.

**Cells**

Primary MCs were generated by extracting bone marrow cells from the femurs of 5–8-wk-old mice and culturing cells in RPMI 1640 medium (Invitrogen) supplemented with 10% inactivated FBS (Thermo Fisher Scientific), 25 mM HEPES (pH 7.4), 4 mM t-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 50 μM 2-ME, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Recombinant murine IL-3 (1 ng/ml; R&D Systems) and recombinant murine stem cell factor (SCF; 20 ng/ml; R&D Systems), both shown to support the in vitro growth and differentiation of the MC precursor, were also included (58, 63). Mouse MCs derived from bone marrow cells with SCF and IL-3 become mature after 4 wk of culture in vitro. We use SCF and IL-3, not WEHI supernatant (supplemented from a murine myelomonocytic leukemia cell line [WEHI-3 cells], rich in IL-3) as a source of MC growth factors to differentiate MCs. After 4 wk, MCs were consistently generated as confirmed by the expression of CD117 (c-Kit) and FcεRI; cell maturation was confirmed by metachromatic staining with toluidine blue. The purity of MCs was greater than 98%. MCs were derived from bone marrow cells of Clnp<sup>−/−</sup>, Thr<sup>−/−</sup> C57BL/6 mice, S1PR2<sup>−/−</sup> BALB/C ByJ mice and their WT littermate mice. Bone marrow cells of S1PR2<sup>−/−</sup> BALB/C ByJ mice and their WT littermate mice were a donation from Dr. Jerold Chun from The Scripps Research Institute, La Jolla, CA.

**MC adoptive transfer into MC-deficient mice**

After culturing a sufficient number of donor MCs from mouse bone marrow cells, we counted cells, resuspended them in an appropriate volume of cold PBS, and kept them on ice until intradermal cell injection (using a 27-gauge needle and a 1-ml syringe), within the back skin of anesthetized Kit<sup>ε-/ε-</sup> mice. The number of total transferred MCs for one recipient mouse was 6 × 10<sup>6</sup> in 400 μl PBS (8 × 50 μl injections in two rows, down the length of shaved back) (63). Cell maturation was confirmed by metachromatic staining with toluidine blue prior to the transfer. A skin biopsy of the recipient mouse was performed to demonstrate that MCs were resident in the skin at the time the experiment was performed after transfer (Supplemental Fig. 1G, 1H). The interval between MC transfer and the infection with VV was 2 wk. The 2-wk time interval is a modification of the original protocol that uses the mice 4–6 wk from the adoptive transfer of the cells.

**Viable cell counts**

We used 0.4% Trypan Blue Stain (Invitrogen), a well-accepted method, for staining dead cells. Trypan blue is a dye that can enter cells with compromised membranes (64).

**Virus**

The purified VV (strain Western Reserve), and recombinant VV vBSR-GFP (65) and anti-L1 7D11 neutralizing mAb for VV (66) have been described previously. BS-C-1 monkey kidney cells were grown to confluence in DMEM medium (Invitrogen) supplemented with 10% FBS for propagation of VV. VV stocks used for infection at 48–72 h after infection of BS-C-1 cells were isolated and purified by sedimentation through two 36% (wt/vol) sucrose cushions followed by one sedimentation on a 25–40% (wt/vol) continuous sucrose gradient; the visible virus band was collected, and virus was pelleted and stored at −80°C. On thawing for experiments, virus was sonicated on ice for 1 min before use (67). Virus plaque assays were performed on human keratinocyte cell line HaCaT or BS-C-1 monkey kidney cells as described previously with supernatant of tissue homogenate or cell culture media (68). Anti-L1 7D11 mAb neutralization assay was performed as described previously (66). Viruses were incubated with 100% plaque reduction titer of 0.01 mg/ml anti-L1 7D11 mAb in DMEM medium (Invitrogen) without serum for 30 min at room temperature before being added to MCs. UVC-inactivated VV was obtained by inactivating VV with UVC (254 nm) radiation dose of 7.6 mJ/cm<sup>2</sup> at room temperature as described previously (69, 70).

**Marine challenge with VV**

The University of California–San Diego IACUC System subcommittee on animal studies approved procedures. The backs of sex-matched adult littermates were shaved, and hair was removed by chemical depilation (Nair; Church and Dwight). Mice were inoculated with 10<sup>6</sup> PFU of vBSR-GFP VV by scarification with 15 pricks into the skin (27, 29). At 3 d after infection, mice were euthanized with CO2 and the lesion areas were pictured alongside with a scale bar. Lesion size was measured using ImageJ (National Institutes of Health) (71). And skin lesions and spleens were collected and homogenized with a Mini-Beadbeater (Biospec Products) for assessing cytokine concentrations by ELISA, VV titer by plaque assay, and...
virus gene expression by quantitative real-time PCR. VV DNA expression was assessed using the same primers that we used for quantifying VV mRNA, but the substrate was the extracted DNA. For cromolyn experiments, WT C57BL/6 littermate mice were injected i.p. with 10 mg/kg body weight of cromolyn sodium salt (Sigma) once every 4 d before experiments. VV was inoculated on the fourth day after cromolyn injection, and cromolyn was still injected i.p. once every day until the mice were sacrificed (72). The mouse experiments were performed on groups of four to nine mice for each treatment and repeated three times.

**Histology**

Animals were sacrificed on the third day of the experiment, and a skin sample of tissue adjacent to the infection wound was collected, fixed with buffered formalin, and embedded in paraffin for H&E staining. Some sections were stained with toluidine blue to identify MCs and MC degranulation (73). If any immature MCs were present in the section, they were not stained by formalin fixation.

**Real-time quantitative RT-PCR**

Trizol Reagent (Invitrogen) was used to isolate total RNA. One microgram of total RNA for cDNA synthesis by the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s instructions was applied for each reaction; it was amplified by real-time RT-PCR in an ABI 7300 Real-Time PCR system (Applied Biosystems). The primers and probes used for real-time RT-PCR were obtained from Applied Biosystems; RNA analysis reagents (TaqMan Master Mix reagents kit) were from Applied Systems. We used the comparative ΔΔCt method to determine the quantification of gene expression, normalized the target gene expression in the test samples to the endogenous reference GAPDH level and reported them as the fold difference relative to GAPDH gene expression in untreated baseline control (74). We performed all the assays in triplicate and repeated the experiments at least three times.

**Early gene expression of VV**

VV early gene expression was evaluated using quantitative real-time PCR normalized to housekeeping gene GAPDH. MCs were cultured in 48-well plates at a concentration of 10^5 cells per well. VV (10^5 PFU) was added to corresponding wells and incubated for 1 h in a total volume of 100 μl, and 500 μl of MC media was added for an additional 24 h. RNA was isolated from cultured cells or homogenized tissues using TRizol (Invitrogen) according to the manufacturer’s instructions. After reverse transcription with the iScript cDNA synthesis kit (Bio-Rad Laboratories), real-time PCR was performed using an ABI 7300 Real-Time PCR system (Applied Biosystems). The following primer sequences were used to assay for the vaccinia gene transcripts: forward, 5'-GCGAAATGGGTTTCCGTTCC-3' and reverse, 5'-AAACACATCCGGCTTCATCATC-3'. This region of the genome encodes a subunit of a DNA-directed RNA polymerase expressed within 2 h of viral Master (75). The TaqMan probe, 6FAM-ATTGAAGTT-CCTCCTCGGCGGTATCGT, was purchased from Applied Biosystems. Samples were amplified in MicroAmp Optical 96-well reaction plates (Applied Biosystems) in a 25 μl volume containing 2X TaqMan Master Mix (Applied Biosystems), 200 nM forward primer, 200 nM reverse primer, 80 nM probe, and template RNA. Thermal cycling conditions were 50°C for 2 min and 95°C for 10 min for the first cycle. Subsequently, samples were amplified for 50 cycles at 95°C for 15 s and 60°C for 1 min.

**Flow cytometry (FACS)**

After treatment with vBSR-GFP VV, MCs were counter-stained with 1 μg/ml propidium iodide (PI; Sigma) on ice according to the manufacturer’s instructions. Cells were analyzed with the Guava EasyCyte 8HT two laser, six-color microcapillary-based benchtop flow cytometer (Millipore) for assessing infection rate and cell viability.

**MC degranulation assay**

Degranulation was assessed by measuring the activity of β-hexosaminidase in the supernatants (76–78) of 1 × 10^5 MCs in 200 μl Tyrode’s buffer (0.1% BSA, 0.1% glucose, 2 mmol/l MgCl₂, 137.5 mmol/l NaCl, 12 mmol/l NaHCO₃, 2.6 mmol/l KCl, pH 7.4) incubated for 1 h with 1 × 10^5 PFU VV, HSV-1, adenovirus, or UVC-inactivated VV. For each sample assayed, supernatant aliquots (20 μl) were mixed with substrate solution (100 μl), which consisted of 1 mM 4-methylumbelliferyl-2-acetamide-2-deoxy-β-D-glucopyranoside (Calbiochem) in 0.1 M sodium citrate buffer (pH 4.5), and were incubated for 30 min at 37°C. The reaction was then stopped by adding 12 μl of 0.2 M glycine (pH 10.7). The reaction mixtures were excited at 365 nm and measured at 460 nm in a fluorescence plate reader (Gemini EM microplate spectrofluorometer; Molecular Devices). To determine the total cellular content of this enzyme, an equivalent number of cells were lysed with 1% triton-X-100 (Sigma). Release of β-hexosaminidase was calculated as the percentage of the total enzyme content, MC stabilizer, cromolyn (1 μg/ml, 5 min at 37°C; Sigma), was used to block MC degranulation.

**MC granule isolation**

To isolate the MC granules and the granule remnants, we followed a classic protocol (79). MC granule release, from C630^-/- MCs and WT MCs, was induced with compound 48/80 (1 μg/ml, 5 min at 37°C). MCs were separated by centrifugation, and the supernatant containing granules and granule remnants were used and treated with VV. For each condition, 1 × 10^6 MCs were used in 1 ml Tyrode’s buffer. Baseline control was the supernatant from MCs treated with PBS. VV stock solution 10 μl (5 × 10^5 PFU) was added to 1 ml solution containing MC granules for 24 h at 37°C. The outcome was measured as VV titer in the supernatant by the plaque assay performed on B5-C1 cells.

**ELISA**

We used ELISA kits (eBioscience) to determine mouse ILS in all supernatants of tissue homogenate or cell culture media according to the manufacturer’s instructions. For tissue samples, we normalized ELISA measurements to total protein content of the sample (BCA Protein Assay Kit; Pierce) (80).

**Fluorescence images**

MCs were stained with 1 μg/ml PI (Sigma) on ice and then attached to a glass slide by using Shandon Cytospin 2 cytcentrifuge (Thermo Fisher Scientific). Prior to cytoplasm, cells were resuspended at 10^6 cells/ml. Each slide had the same amount of spun cells. Slides were mounted in ProLong Anti-Fade reagent with DAPI (Molecular Probes). For autofagy assay, we used LC3B Ab Kit for autophagy (Invitrogen) according to the manufacturer’s instructions. We imaged the MCs using the Bx51 research microscope (Olympus) and X-Cite 120 fluorescence illumination systems (EXFO Photonic Solutions).

**Golgi membrane separation**

The Golgi membrane separation was done by gradient ultracentrifugation as described previously (81). The whole homogenate of HaCaT cells (human keratinocyte line) is adjusted to 1.4 M sucrose (Sigma) and layered under a discontinuous sucrose density gradient of 1.2 and 0.8 M sucrose. After centrifugation for 2 h in an ultracentrifuge at 110,000 × g and 4°C, the Golgi membranes, which have a much lower density than those of the other membrane particles, float upward to band at the 1.2/0.8 M sucrose interface.

**Autophagy assay for MC**

We used the LC3B Ab Kit for Autophagy (Invitrogen) according to manufacturer’s instructions.

**SIP signal blocking assay**

An S1PR2-specific antagonist, JTE013 (10 μM, Tocris Bioscience), was added to MCs 30 min before virus infection (multiplicity of infection [MOI] = 1). SIP (20 μM, Cayman Chemical) was added to MCs immediately after virus inoculation. The IC50 = 22 nM of JTE013 is used for human and rat receptors. However, in mouse MCs, most research used JTE013 = 10 μM (82). In some experiments, desipramine (10 μM, Sigma), an inhibitor of acidic sphingomyelinases, and N,N-dimethylsphingosine (10 μM; Cayman Chemical), a competitive inhibitor of sphingosine kinase, were added to MCs 30 min before virus infection.

**Dot blot analysis**

Supernatants from in vitro MC culture were collected 24 h after VV infection (MOI = 1), and 100 μl was applied to a nitrocellulose membrane (Whatman) using S&S Minifold I Dot-Blot System (Schleicher and Schuell). The membrane was blocked in Odyssey Blocking Buffer (LI-COR) and then incubated with a rabbit polyclonal anti-CRAMP Ab (0.25 μg/ml; Abs against the CRAMP peptide were prepared by Quality Controlled Biochemicals, Hopkinton, MA) in Odyssey Blocking Buffer for 1 h. Incubation with the secondary Ab, IRDye 680 Goat anti-rabbit secondary Ab (LI-COR) diluted 1:15,000 in Odyssey Blocking Buffer, was performed for 1 h. Immunoreactivity was visualized using an Odyssey Infrared Imaging System (LI-COR) according to the manufacturer’s instructions. All incubations were performed at room temperature.
Statistical analyses

All data are presented as mean ± SD. At least three independent experiments were performed to assess the reproducibility of the different experiments. The two-tailed t test and one-way or two-way ANOVA with Bonferroni’s post-test of GraphPad Prism Version 4 were used to determine significance between two groups or multiple groups. For all statistical tests, p values < 0.05 were considered statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001).

Results

Mast cell deficient mice are more susceptible to VV infections

To study the role of dermal MCs in combating skin-initiated viral infections, we infected MC-deficient mice (Kitwsh2/2) and WT littermates (C57BL/6) with VV. Mice were scarified and 10^6 PFU of VV was applied on the lower back. The Kitwsh2/2 mice developed lesions at 24 h, and the lesions increased in size over time while the WT littermates developed only a few pinpoint lesions at 3 d after infection (Fig. 1A, 1B). A histologic section of the WT skin at the inoculation site showed a significant increase in the number of MCs (Supplemental Fig. 1A), thereby implicating MCs in the viral response.

Our evaluations were based on the wound development after viral scarification. Because it was reported previously (83) that abnormal wound repair occurs in Kitwsh2/2 mice, we also ruled out the possibility of an abnormality in the scarification healing of Kitwsh2/2 mice, by performing in parallel, the scarification of Kitwsh2/2 mice without virus application. In our scarification model, Kitwsh2/2 mice displayed normal scarification repair (Supplementary Fig. 1B).

To ensure that our observation was not related to the particular strain of mice that we were using, we also repeated the experiment using a second MC-deficient model such as the W/W-v. As you can see in Fig. 1H–M, we obtained overlapping results, clinically (Fig. 1H–L) and biologically (Fig. 1M).

Reconstituted MC-deficient mice have normal susceptibility to VV infections

To confirm that the susceptibility of Kitwsh2/2 mice to VV was directly related to MC activity and not to other unknown defects present in the mice, we used an adoptive transfer mouse model. MC-deficient mice were used as recipients of MCs derived from WT littermates. MCs derived from the bone marrow of WT lit-
terminals were differentiated in culture and injected intradermally into the Kitwsh−/− mice upon maturation. A skin biopsy was performed to demonstrate that MCs were resident in the skin at least 2 wk after transfer (Supplemental Fig. 1F–J). After 2 wk, the reconstituted Kitwsh−/− mice and two control groups, including nonreconstituted MC-deficient mice and WT littersmates, were scarified in the presence of 10^5 PFU of VV. The MC-deficient mice reconstituted with WT MCs had significantly smaller lesions than the nonreconstituted mice (Fig. 1C). The lesion size was quantified with National Institutes of Health ImageJ software (71, 84) and expressed in square millimeters (Fig. 1D). After 3 d, the lesioned skin and spleens were excised and processed in TRIzol for RNA extraction to determine viral replication. We quantified the early expression of VV (DNA-directed RNA polymerase mRNA, which encodes a gene subunit expressed within 2 h of viral entry) in the wound site and spleen by real-time quantitative PCR. Kitwsh−/− mice had a significantly higher expression of viral RNA compared with WT littersmates (Fig. 1E, 1G). To determine whether early gene expression was followed by virus replication, we harvested and homogenized the tissue and seeded the supernatant on BS-C-1 cells to count plaque formation (Fig. 1E). Kitwsh−/− mice had a significantly higher level of infectious virus than WT littersmates. Mast cell reconstituted Kitwsh−/− mice showed similar levels of infectious virus as compared with the WT littersmates that correlated with the wound clinical presentation (Fig. 1E). The reconstituted mice showed viral levels similar to those in the WT littersmates, as the MC reconstitution of Kitwsh−/− mice prevented the spreading of the virus to the circulation and the spleen (Fig. 1G). We studied the number of MCs at the sites of inoculation (Supplementary Fig. 1J) and their capacity to degranulate in WT and Kitwsh−/− reconstituted mice (Supplementary Fig. 2A, 2B). The results showed no differences between reconstituted and WT littersmates. A panel of proinflammatory and anti-inflammatory cytokines was tested, and TNF-α, IL-6, and IL-10 showed significant changes. The reconstituted mice showed a higher level of TNF-α (columns 1, 2, and 3 versus 4, Supplemental Fig. 1C) and a lower level of IL-10 (columns 1, 2, and 3 versus 4, Supplemental Fig. 1E) as expected. Levels of IL-6, a proinflammatory cytokine, increased in all the infected groups (columns 1, 2, and 3 versus 4, Supplemental Fig. 1D) when compared with noninfected groups, but they were much higher in the Kitwsh−/− nonreconstituted infected mice (Supplemental Fig. 1C–E). Kitwsh−/− mice have been reported to have myeloid and megakaryocyte hyperplasia, which could have explained the defective immunity noticed in the mice after viral challenge (60); however, because reconstitution with MCs promoted normal viral response, we concluded that the defect observed was primarily due to the absence of MCs. The absence of MCs impairs the capacity of the skin to respond to VV infections and can be corrected by the reconstitution of MCs in the skin.

MC degranulation reduces the severity of VV infection

On the basis of the in vivo data, we next sought to determine the mechanism of MC antiviral action. We hypothesized that MCs can act against pathogens through their ability to degranulate and release antimicrobial molecules. We demonstrate that MCs are resistant to VV infection up to an MOI of 0.1 (Fig. 2A–C), and they begin to degranulate on contact with VV as evidenced by β-hexosaminidase release (1 h observation in Fig. 2D). The addition of cromolyn, a well-described inhibitor of MC degranulation (5) (Fig. 2D), increased viral susceptibility and resulted in an increased VV-induced cell death (Fig. 2E). Cromolyn itself has no inhibitory activity on VV (Supplemental Fig. 2C).

To provide better insight into the role of MC degranulation in viral immunity, we investigated the antiviral activity of MC granules in vivo. We pretreated WT littersmates with cromolyn sodium for 4 d before VV application and every 24 h for the duration of the experiment. Mice pretreated with cromolyn developed a lesion that was comparable in size to the Kitwsh−/− mice lesion and significantly larger than lesions on the WT littersmates (Fig. 2F–H). Viral replication was also higher in the cromolyn pretreated when compared with untreated (Fig. 2I–J), suggesting that MC degranulation attenuates VV infection.

VV envelope fusion induces MC degranulation independent of TLR2

Because these observations demonstrate that MCs respond to VV infection, we next wanted to determine how MCs degranulate upon encountering VV. We hypothesized that the VV envelope contains the signal for MC degranulation. Because the envelope contains many different components, partly from cells and partly specific to the virus, we tested our hypothesis in different ways. First, to rule out that cell contaminants were responsible for inducing degranulation, we used purified VV (Fig. 3A) in comparison with unpurified VV and we showed that there are no differences in degranulation. Second, we used short-wavelength UVC (254 nm) inactivated VV (the virions lack infectivity, but not fusion capacity with the cells (85, 86) (Fig. 3A). We demonstrated that UVC inactivated VV still induced cell degranulation (Fig. 3A). Third, we blocked one viral membrane protein L1 essential for viral fusion with a specific neutralizing Ab to L1 (Fig. 3A). In the presence of the neutralizing Ab the cells did not degranulate, suggesting that the function of the L1 protein in mediating virus entry is required for MC degranulation.

When VV enter cells by membrane fusion, sphingomyelin and other phosphatidyserine lipids in VV envelopes can be discharged in the MC membrane; the discharge will lead to a chain of events that includes the formation of S1P and activation of S1PR2. This final event stimulates MC degranulation (42–44). To verify that this is the exact pathway involved in MC-VV degranulation, we added S1PR2 receptor-specific antagonist JTE013 (87) to MCs before VV inoculation. JTE013 also blocks S1PR4 in other cell types, but MCs lack this receptor (44). We show that MCs treated with JTE013 failed to degranulate in response to VV (Fig. 3B); moreover, they were more susceptible to virus-induced cell death (Fig. 3C) and exhibited a higher viral load (Fig. 3D). The exogenous addition of S1P rescued the effect of the JTE013 block (Fig. 3B–D). And the removal of JTE013 from the medium reverted the capacity to respond to VV, demonstrating that MCs were specifically blocked but not damaged. Because the use of JTE013 at the required blocking concentration could raise the question of the specificity of its effects, we also challenged S1PR2 knockout MCs (S1PR2−/−) with VV (Fig. 3E) and demonstrated that these cells were more sensitive to viral infection. To support previously discussed results, the infection (Supplemental Fig. 3C) was proportional to the amount of degranulation (Supplemental Fig. 3D). In Supplemental Fig. 3E, we demonstrated that in addition to blocking S1PR2 with JTE013, the block of sphingosine kinase (the enzyme that generates S1P) with N,N-dimethylsphingosine significantly increased the percentage of infected MCs, which can be rescued by addition of S1P, whereas inhibiting acidic sphingomyelinase, a nonmembrane enzyme involved in sphingomyelin metabolism, with desipramine does not increase VV infectivity in MCs. Because sphingomyelin is present in both viral membranes and Golgi membranes, we isolated Golgi membranes and demonstrated that they were able to induce degranulation in a dose-dependent manner (Fig. 3A, Supplemental Fig. 3B). We also
showed that HSV-1 enveloped virus induced MC degranulation (Supplemental Fig. 3A); however, adenoviruses, which are non-enveloped viruses, failed to induce a significant amount of degranulation (Supplemental Fig. 3A).

TLR2, one of the pattern recognition receptors in the skin, has been previously implicated in VV infectivity and cytokine responses in different cell types (88, 89). To investigate whether TLR2 was important for VV-induced degranulation in MCs, we

FIGURE 2.  Mast cells degranulate upon contact with VV. A, Mast cells were infected with VV at different MOI for 24 h and analyzed by FACS. The right plot shows resting MCs before virus interaction; the two other plots show cells after contact with VV. The upper quadrant number is the percentage of infected MCs. B, FACS analysis of VV expression in MCs infected with VV (GFP+ cells) at different MOI. The inset shows green fluorescence in MCs infected with VV. The nucleus was stained in red with PI. C, VV titer 24 h after coculture VV with MCs at different MOI. VV titer in the supernatant was expressed as PFU by plaque assay in BS-C-1 cells (original magnification ×400). D, Mast cell β-hexosaminidase release (as index of granule content release) 1 h after contact with VV. The granule release was blocked by adding cromolyn (cro) to the medium. **p < 0.01 compared with other groups. E, MC death was measured with trypan blue 1 h after contact with VV. The number of dead cells was increased by cromolyn in the medium. All the in vitro experiments have been performed in triplicate. **p < 0.01. F, WT C57BL/6 mice at 72 h after VV inoculation. G, Cromolyn pretreated WT C57BL/6 mice at 72 h after VV inoculation. H, Quantification of skin lesion size (mm²) in nonpretreated and cromolyn-pretreated mice at 72 h after infection. I, Quantification of viral load in skin lesions expressed as PFU/ml. J, Quantification of VV early gene expression in skin lesions by real-time quantitative RT-PCR. Three independent experiments with four mice per group were performed for each experiment. ***p < 0.001.
used Tlr2−/− MCs. MCs were derived from the bone marrow of WT and Tlr2−/− mice. WT and Tlr2−/− MCs were efficiently infected by VV, and their capacity to degranulate was equivalent to the WT cells (Fig. 3F). These data suggest that TLR2 is not necessary for VV-induced degranulation in MCs.

Expression of cathelicidin (mCamp) is critical in MC defense against VV in vivo

We have demonstrated that MCs function through degranulation to attenuate VV infection. We next wanted to investigate how degranulation influences the MC antiviral capacity. Cathelicidin antimicrobial peptides, previously shown to have antiviral activity in vitro and in vivo (90, 91), are expressed by MCs in vivo (6). We demonstrated that cathelicidin (mCamp) is released during VV-induced degranulation (Fig. 4A). Notably, in the cromolyn-pretreated mice, the mCamp levels at the site of the infection were lower than in the non-pretreated mice (Fig. 4B). A similar low level of mCamp was found in nonreconstituted Kitwsh−/− mice at the site of VV infection in skin when compared with WT littermates (Fig. 4C). To verify the biologic relevance of these findings, we reconstituted Kitwsh−/− mice with Cnlp−/− MCs (deficient in cathelicidin antimicrobial peptides) or WT MCs. Wound development and viral load were compared after viral challenge. The Kitwsh−/− mice reconstituted with WT MCs had a reduced viral load and reduced wound development when compared with Kitwsh−/− mice reconstituted with Cnlp−/− MCs as assessed by the wound size measurements (Fig. 4D–H). This correlated with VV titer from the wound sites (Fig. 4I). Moreover, Kitwsh−/− mice reconstituted with Cnlp−/− MCs also showed an increased VV expression in the skin (Fig. 4I) and levels of keratin (Fig. 4J) and elevated levels of skin IL-6 (Fig. 4K). Only reconstitution with WT MCs maintained skin IL-6 levels comparable to WT littermates (Fig. 4K). The observed differences were not due to a different level of cell infiltration in the lesions (Supplemental Fig. 4A–C.)

To investigate whether cathelicidin expression in MCs is directly coupled with their antiviral response, we compared the infectivity of Cnlp−/− MCs to WT MCs. We derived MCs from the bone marrow of WT and Cnlp−/− mice and infected with VV. Cnlp−/− MCs were more sensitive to virus compared with WT MCs. They demonstrated more viral RNA and DNA (Fig. 5A, 5B), a higher VV titer in the supernatant and more VV infected GFP+ cells when compared with WT (Fig. 5C–E). To further confirm that the Cnlp−/− MCs’ impaired capacity to kill VV was linked to their granule content and release, MCs were pretreated with or without cromolyn sodium. As illustrated in Fig. 5F, Cnlp−/− cells have no advantage from the release of their granules upon viral encounter. In addition, purified Cnlp−/− MC granules fail to kill VV, further demonstrating that the effects observed are not due to differences in granule release (Fig. 5G). The WT granules are directly killing
VV, a dose-dependent effect that had not been observable with granules from Cnlp<sup>−/−</sup> MCs (Fig. 5G). Cnlp<sup>−/−</sup> MCs in vitro characterization is presented in Supplemental Fig. 4D–H and shows that granule composition in TNF-α and metachromasia is similar between Cnlp<sup>−/−</sup> and WT cells and that there were no changes in the number of degranulating cells or in autophagy formation on viral contact. When examining at 72 h after VV infection in vivo, the presence of Cnlp<sup>−/−</sup> MCs is still an advantage over having no MCs at all (Fig. 4I,4J).

**Discussion**

We have demonstrated fundamentally new insights into VV immunity. First, MCs can sense VV and resist infection proportionally to the viral load. Second, MCs degranulate on VV fusion via interaction with the viral lipid envelope. Third, the response to VV in skin is substantially mediated by degranulation and cathelicidin granule expression in MCs. To our knowledge, this study is the first to describe the direct ability of MCs to elicit an antiviral response through antimicrobial peptide activity.

MCs have been long regarded as essential resident effector cells in the elicitation of the allergic response through the secretion and generation of bioactive mediators. In the last few years, their nature as defense cells has been exploited. However, their antimicrobial activity has been primarily regarded as secondary to their activation of other host defenses such as neutrophil activation. In the past there have been a few reports that suggested a role for MCs in the initiation of chemokine-dependent host responses to virus infections, and of histamine release in response to viral contact (45, 51, 52); however, the direct capacity of MCs to kill VV through antimicrobial peptides has not been reported before (45–50). Our data provide evidence that MCs are directly involved in host defense against viral infections. MC-deficient mice were signifi-
significantly more susceptible to viral infections as they developed larger wounds at the site of infection and had higher viral gene expression. Mast cell reconstitution reverted the phenotype to a normal response even after only 2 wk from the reconstitution with in vitro matured cells. This in vivo phenomenon could be explained by either MCs directly killing the virus at the port of entry or MCs releasing chemokines that mediate cell chemotaxis. However, according to our in vitro data, MCs directly kill viruses through degranulation. Many different cytokines have been studied in viral infections. The most representative and best studied for their interaction with VV are TNF-α, IFN-γ, IL-1β, IFN-β, IL-12, IL-6, and IL-10 (92). We therefore chose to study these cytokines and their relationship with MCs during a viral infection. We reported only the cytokines that showed any change in one or more mice groups, which were then selected for further study. We examined cytokine protein level by ELISA. MC-derived TNF-α has a crucial role in the influx of neutrophils into lesions. In this study, we observed that VV induced IL-6 and TNF-α secretion, especially in MC-deficient mice. On the contrary, MC presence decreases the viral load in the tissue affected by VV infection. IL-6 and TNF-α levels correlated with the clinical presentation of our infected mice, such that higher levels of IL-6 and TNF-α occur in more
severe infections. Therefore, the absence of MCs amplifies the inflammation. IL-6 MC production has been recently implicated in cancer response (11), but levels of IL-6 do not usually increase in VV infections (93). The fact that, in our experiments, IL-6 levels increase in the absence of MCs, indicates a role for MCs in controlling the level of this IL in viral infections and inflammation at large. IL-10 is an anti-inflammatory cytokine that has recently been in the center of some MC debates (63). The absence of IL-10 when MCs are not present is considered to make lesions worse (57). We demonstrated that IL-10 levels decreased in the presence of VV in both the reconstituted and nonreconstituted Kitwsh−/− mice, indicating that the difference was not specifically due to variations in MC presence.

The MC degranulation process has been long regarded as secondary to the binding of IgE allergen-specific Abs. Recently, innate immune receptors such as TLRs were identified on the surface of MCs, which could allow MCs to directly recognize pathogens (94). In this study, we hypothesized that MCs react to VV on contact and fusion is probably the mechanism that triggers degranulation. Our in vitro data demonstrated that MCs, which fail to degranulate on viral contact, had higher viral titers and more cell death. This finding was also observed in vivo where mice with MCs that could not degranulate were more prone to infection. Pretreatment with cromolyn sodium blocked MC activation and degranulation, creating a phenotype similar to MC-deficient mice during a VV infection. Cromolyn is a chromone medication that has been available for decades to manage allergic disorders. It has been used as an inhaler for asthma, a nasal spray for allergic rhinitis, eye drops for allergic conjunctivitis, an oral liquid for mastocytosis and food allergy, and an ointment for atopic dermatitis. Despite intensive research in many laboratories over the last 40 years, a precise mechanism to explain the clinical activity of cromolyn has not been confirmed. However, the primary mode of action of cromolyn was thought for many years to be the stabilization of MCs and thus preventing subsequent release of mediators following appropriate challenge (95–97). We demonstrated that cromolyn inhibited MC degranulation in vitro, whereas in vivo other mechanisms could be present amplifying MCs direct activity against viruses.

The mechanism of VV entering cells is not precisely known. Previous reports indicated that VV enters cells through different routes including endocytosis (30, 31) and plasma membrane fusion (32–36, 98). Moreover, VV entry pathways and signaling differed depending on cell types (33, 98–102) and viral strains (37, 103). These studies revealed a complex relationship of VV entry processes with host cells. Bisht et al. (104) demonstrated that the L1 protein is required for membrane fusion and entry. Using a specific mAb against the L1 7D11 fusion protein, we demonstrated that viral envelope fusion is required for MC degranulation. The study was also supported by UVC-treated virions that are able to fuse but not to be infectious. The VV membrane contains a variety of phospholipids (41). In a series of experiments, Ichihashi et al. (105) showed that the loss of VV infectivity on Nonidet P-40 detergent extraction of lipids could be partially rescued by incubation of the extracted virions with exogenous lipids, including crude mammalian cell membrane preparations. Additional studies implicated phosphatidylserine in VV entry (39, 106). We demonstrated a similar mechanism with respect to MC viral recognition and degranulation. Because VV envelopes discharge lipids on the MC membrane surface, we hypothesized that the sphingomyelin–ceramide–sphingosine–S1P membrane pathway was implicated in the activation of the degranulation process. This pathway ends with the production of S1P to stimulate S1P2 (44). We demonstrated that blocking this receptor significantly reduced the degranulation response on viral contact. It has been previously recognized that degranulation of MCs derived from bone marrow of S1PR2 knockout mice is impaired (107); we confirmed this observation and we demonstrated that the cells derived from the knockout S1PR2 mice were more susceptible to VV infection. This mechanism is important for blocking enveloped viruses because it gives MCs the chance to kill as many viruses as possible before they infect the cells.

In our in vitro model, MCs degranulated when we used Golgi membranes even without the presence of viral proteins; however, the quantity of lipids present in the Golgi preparation is extremely high in sphingomyelin and may generate S1P even without the necessity of fusion according to the hypothesis of the sphingolipid rheostat that activates cell membrane (108). Degranulation on envelope contact was not specific to VV, because other enveloped viruses (e.g., HSV-1) induced degranulation while adenovirus did not. Therefore, we can hypothesize that other enveloped viruses are recognized by MCs through fusion and lipid discharge.

Other mechanisms are certainly in place after the cells have been infected. MCs are equipped with TLR3, TLR7, and TLR9 intracellular receptors and the capacity of further responding to viral infections (2, 9); however, our data suggest that degranulation constitutes the first cellular lines of defense against VV. Thus, the composition of the granules becomes essential to the antiviral capacity of the cell. Antimicrobial peptides are an evolutionarily conserved component of the innate immune response and are found among all classes of life. Two antimicrobial peptide gene families expressed by mammalian mucosal epithelia, Cathelicidin and defensins, have received the most attention (109–111). They are both expressed in MCs. However, MC antimicrobial function against bacteria in skin is mediated by cathelicidin activation (58). Cathelicidin has been shown to have an in vitro and in vivo direct effect against viruses (21, 90, 112). Unlike the antibacterial activity of this peptide, the antiviral activity is not fully dependent on disulfide bond formation. Viral inhibition appears to result, in part, from disruption of the envelope and/or capsid (21).

We demonstrated that the granule-derived antimicrobial peptide cathelicidin is critical for the MC response against VV. We first described that in the absence of MCs, mouse cathelicidin was minimally expressed at the site of infection, even 72 h after the inoculation. The absence of MCs caused a lack of antimicrobial peptide presence either directly the result of the absence of MC cathelicidin or indirectly the result of the capacity of MCs to recruit other cell types producing antimicrobial peptides. In fact, in VV infections neutrophil recruitment begins from day 2 after VV inoculation and reaches peak on day 10 (113–115). In the case of our experimental model of VV infection, cathelicidin is critical for its direct effect on defense during the initial phase of the infection (mice reconstituted with Cnlp−/−MCs have bigger lesions), but is not critical for the recruitment of other cell types like neutrophils; our data show no differences in the cell infiltration at 72 h between mice reconstituted with WT MCs and mice reconstituted with Cnlp−/− cells. However, the presence of VV in the spleen suggests that the initial window phase is critical for the systemic distribution of VV. This finding led us to confirm these results in vitro and further investigate the mechanism. Our data indicate that Cnlp−/−MCs have a significantly decreased ability to suppress VV proliferation. We demonstrated that Cnlp−/−cells that lack cathelicidin were more susceptible to VV in vitro.

Cathelicidin is reduced in patients with atopic dermatitis, and cathelicidin deficiency is linked to the susceptibility to infection with VV and herpes simplex viruses (21, 90, 116). Our data might explain why a disease with normal MC number but low cathelicidin levels has an increased susceptibility to VV infections.
Cathelicidin functions primarily as an antimicrobial; however, it has been implicated in other immune functions (20); therefore, we had to rule out any indirect antiviral activity that cathelicidin might have on MC function. It has recently been described that autophagy is dependent on cathelicidin presence in macrophages (117). As such, we also investigated autophagy formation during a VV infection in Cnlp−/− MCs to rule out changes in cellular physiology. As reported in our supplemental data, we did not identify any difference in autophagy formation or in the number of degranulated cells during a VV encounter.

In this study, we provide evidence that MCs intervene in VV infections. Current literature supports the concept that MCs have a relevant role in fighting infections in general, and our data confirm that MCs have an important role in blocking VV pathways. Our findings suggest that the expression and control of MC antimicrobial peptides is key to this transformation. Therefore, any agent or disease that interferes with the capacity of MCs to release their granules, such as the MC stabilizers cromolyn sodium or steroids, will interfere with the capacity of MCs to block pathogen invasion. This work has the potential to provide significant new insights into the pathogenesis of skin infections and change the way we understand the role of MCs in blocking viral infections in the skin. We provide evidence that MCs interact with VV; however, the mechanisms identified by this study may be applicable to any other enveloped viral infections, and the knowledge obtained from this work will have several therapeutic applications for combating viral diseases.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Information

Figure S1, related to Figure 1.

Figure S2, related to Figure 2.

Figure S3, related to Figure 3.

Figure S4, related to Figure 4 and 5.
Figure S1, related to Figure 1. Mast cell reconstitution of Kit$^{wsh-/}$ mice. (A) Mast cell was counted in randomly selected fields (X200) of baseline control and 72 hours after VV inoculation. ***P<0.001. (B) Wild type and Kit$^{wsh-/}$ mouse skin present no difference after scarification without VV. Our protocol requires superficial scarification. Baseline control was wild type and Kit$^{wsh-/}$ mice scarified without VV application. As visible in the image of Kit$^{wsh-/}$ mouse scarified without VV do not differ from wild type mouse and do not develop spontaneous wound in the absence of VV. The figure shows the scarification area after 72 hours for wild type and of Kit$^{wsh-/}$ mouse.

Cytokines expression in the tissue homogenate from skin lesions of wild-type mice, Kit$^{wsh-/}$ mice and Kit$^{wsh-/}$ mice reconstituted with wild-type mast cells 3 days post-infection with $10^6$ PFU VV. (C) Mouse TNF-α ELISA quantification. Infected tissue showed no difference in TNF-α levels, while TNF-α was significantly increased in all of VV infected mice without regard to their mast cell status compared to non-infected mice. ***P<0.001. (D) Mouse IL-6 ELISA quantification. IL-6 was dramatically increased in the mast cell deficient non-reconstituted mice; and it was also significantly increased in all of infected mice when compared to non-infected mice meaning that its increase is related to the severity of the infection. ***P<0.001. (E) Mouse IL-10 ELISA quantification. IL-10 was significantly decreased in all of infected mice when compared to non-infected mice. ***P<0.001. Please note the unit of cytokines protein is pg/μg total protein, not pg/ml as usually used. So the value of
cytokines protein is much smaller, but more accurate for measuring cytokines protein in solid tissue samples such as skin (Lira et al., 2009). (F) C57BL/6 wild type littermate mouse (mast cells in dermis are indicated by arrows). (G) Kit<sup>wash−/−</sup> mouse skin section (there are no mast cell in the dermis). (H) Kit<sup>wash−/−</sup> mouse reconstituted with mast cells stained with toluidine blue 2 weeks after injection into the skin (mast cells in dermis indicated by arrows). (I) Kit<sup>wash−/−</sup> mouse skin section 72 h after vaccinia virus infection. Still mast cells are not visible in the section. (J) Mast cell was counted in randomly selected fields (X200) in the dermis of the scarification area in baseline control without VV infection and mice 72 h after VV inoculation in Wild type and Kit<sup>wash−/−</sup> mice reconstituted with wild type MCs and Cnlp−/− MCs. There is no difference in the mast cell number in skin of WT mice and Kit<sup>wash−/−</sup> mice reconstituted with wild type or Cnlp−/− mast cells before and after VV infection. Three independent experiments with 5 mice per group were performed for each experiment.

Figure S2, related to Figure 2. In vivo Mast cell degranulation. (A) Mast cells were determined by morphometry (high power 400X, mast cells stain metachromatically with toluidine blue). Mast cells were defined as "degranulated" when more than eight metachromatic extracellular mast cell granules were detected by light microscopy (Paus et al., 1995). (B) Mast cell was counted in randomly selected fields (X200) of baseline control and 72
hours after VV inoculation. No significant difference in mast cell degranulation 
in situ in the skin between C57BL/6 wild type littermate mice and Kit<sup>whsh-/-</sup> mice reconstituted with mast cells. (C) Cromolyn does not directly affect VV. The graph shows the VV survival in PFU. ***P<0.001. Three independent experiments with 5 mice per group were performed for each experiment.

**Figure S3, related to Figure 3. Mast cells degranulate in contact with viral envelopes.** (A) β-hexosaminidase release of mast cells upon contact with purified live VV (p-VV), HSV-1 and adenovirus at MOI=1 for 1h. 48/80 is the positive control. NS: no significant difference between vehicle and Adenovirus treatment. **P<0.01 compared to other treatments except for treatment with Adenovirus. (B) Dose-dependent mast cell degranulation by golgi membrane. Different dilution of separated golgi membranes induce degranulation proportionally to the relative concentration. *P<0.05, **P<0.01. (C) FACS analysis of S1PR2<sup>-/-</sup> MCs generated from bone marrow of Balb/C ByJ background S1PR2<sup>-/-</sup> mice and their wild-type littermate mice. Mast cells were inoculated with GFP<sup>+</sup> VV at MOI=1 for 24 hours. Left quadrants show the percentage of MCs infected by VV. (D) β-hexosaminidase analysis of granule release was determined in mast cells generated from bone marrow of S1PR2<sup>-/-</sup> Balb/C ByJ mice and their wild-type littermate mice. **P<0.01. (E) FACS analysis of GFP<sup>+</sup> MCs infected by GFP<sup>+</sup> VV. **P<0.01 compared to other
groups. Each experiment has been performed in triplicate.

**Figure S4, related to Figure 4 and 5. (A-C)** Hematoxylin and eosin staining (H&E) of skin section of WT mice and Kit$^{wash-}$ mice reconstituted with wild type MCs and Cnlp$^{-/-}$ mast cells of baseline control without VV infection and 72 hours after VV inoculation at 200X. (A) Baseline control (B) 72 hours after VV inoculation. (C) The number of infiltrated cells was counted in randomly selected high power fields (×400). No difference in cell infiltration was noted between wild type and reconstituted mice. Three independent experiments with 5 mice per group were performed for each experiment.

**Cnlp$^{-/-}$ mast cell characterization: (D-H)** (D) Cnlp$^{-/-}$ mast cell metachromasia is similar to wild type MCs. (E) Cnlp$^{-/-}$ mast cell TNF-$\alpha$ response to stimula is similar to wild type MCs. (F) FACS analysis of mast cell degranulation by annexin-V Phycoerythrin (PE) staining (Demo et al., 1999; Suzuki and Verma, 2008). The ability of mast cell degranulation in response to VV was evaluated by adding VV at MOI=1 to the cell cultures and evaluated at 1 and 24 hours. Upper quadrants show the percentage of cells that degranulated in response to VV. (G) $\beta$-hexosaminidase analysis of granule release was determined in wild-type C57BL/6 MCs and Cnlp$^{-/-}$ MCs in the presence or absence of different degranulation conditions. The release of $\beta$-hexosaminadase of Cnlp$^{-/-}$ MCs was similar to wild type cells. (H) Autophagy in mast cells. LC3B antibody (red) was
used to detect signals of autophagy in the presence of VV at MOI=1. Mast cells counterstaining was done with DAPI (blue). No difference were noticed between Cnlp−/− and wild type mast cells. Each experiment has been performed in triplicate.
Figure S1, related to Figure 1.

**A**

![Graph showing mast cell number](image)

**B**

*Figure showing images of C57BL/6 wild type littermate mice and Kit^wsh/wsh^− mice with and without WV treatment.*

**C**

![Graph showing mRNA levels](image)

**D**

![Graph showing mIL-6 levels](image)

**E**

![Bar chart showing mIL-10 levels](image)

**F, G, H, I**

*Images showing histological sections with annotations.*

**J**

![Scatter plot showing mast cell number](image)
Figure S2, related to Figure 2.

A

mast cell degranulated

CS7BL/6 wild type littermate mice

Kit<sup>W-sash/-</sup> mice reconstituted with wild type mast cells

B

Degranulated MC number (per field)

WT
KT+WT MC
KT+Clnlp/- MC
KT+VV
KT+WT MC+VV
KT+Clnlp/- MC+VV

C

Total vaccinia virus in supernatant (PFU)

PBS
Cromolyn
CRAMP
Figure S3, related to Figure 3.
Figure S4, related to Figure 4 and 5.

A. Wild type mice, KIt^a^b^c^ mice reconstituted with WT mast cells, and KIt^a^b^c^ mice reconstituted with Cnlp^-^ mast cells.

B. Wild type mice, KIt^a^b^c^ mice reconstituted with WT mast cells, and KIt^a^b^c^ mice reconstituted with Cnlp^-^ mast cells.

C. Cell infiltration in skin (field).

D. C57BL/6 wild type mast cell and C57BL/6 Cnlp^-^ mast cell.

E. 10^5 mast cell + 10^6 PFU vaccinia for 12 hr.

F. Degranulation (Amunin V PB) 1 hr after PBS, 1 hr after VV, 24 hrs after VV.

G. % of l-lexosaminidase release (total).

H. Wild type mast cell and Cnlp^-^ mast cell.