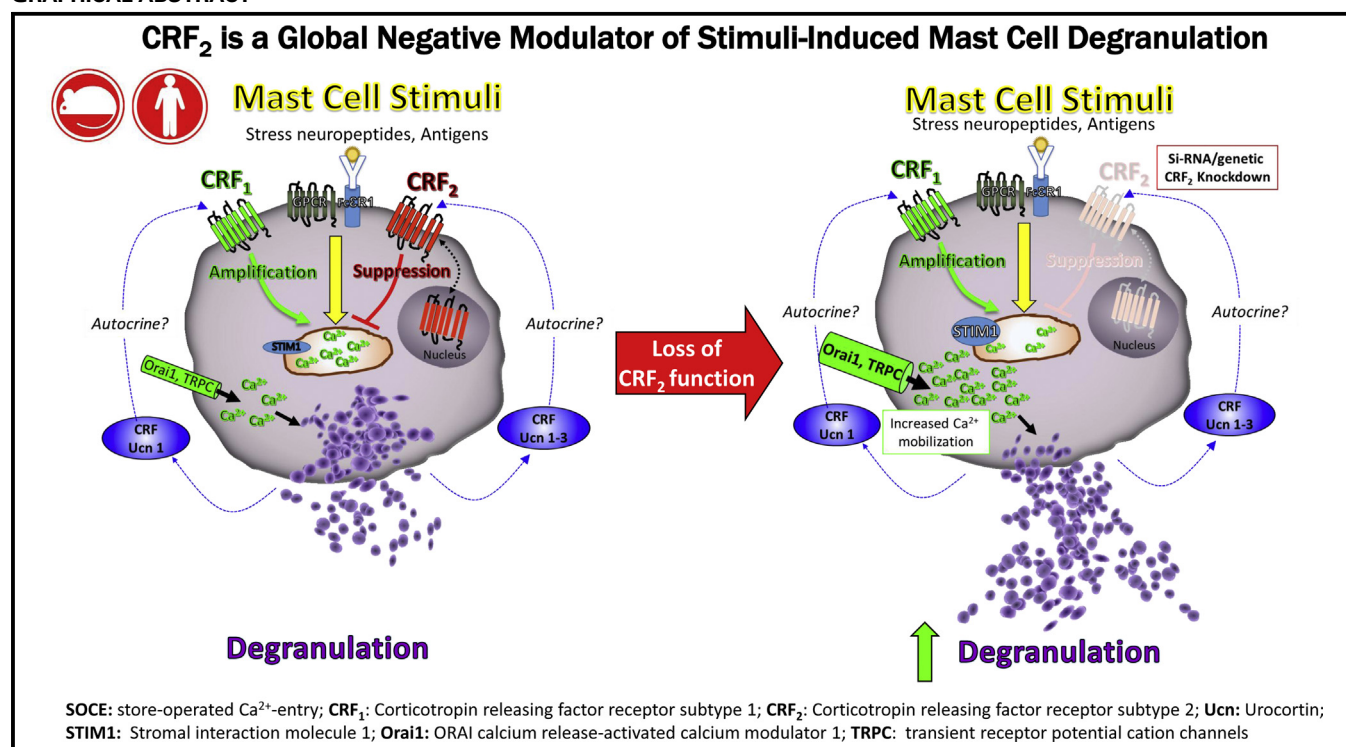


Mast cell corticotropin-releasing factor subtype 2 suppresses mast cell degranulation and limits the severity of anaphylaxis and stress-induced intestinal permeability



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GRAPHICAL ABSTRACT



Background: Psychological stress and heightened mast cell (MC) activation are linked with important immunologic disorders, including allergy, anaphylaxis, asthma, and functional bowel diseases, but the mechanisms remain poorly

defined. We have previously demonstrated that activation of the corticotropin-releasing factor (CRF) system potentiates MC degranulation responses during IgE-mediated anaphylaxis and psychological stress through corticotropin-

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Supported by US National Institutes of Health grants R01 HD072968 (to A.J.M.), R21 AI140413 (to A.J.M.), K08/R03 DK097462 (to A.J.M.), R01 DK080787 (to A.B.), and F30 OD025354 (to E.M.). A.J.M. is the Matilda R. Wilson Endowed Chair of Large Animal Clinical Sciences at Michigan State University, College of Veterinary Medicine. Astressin 2B was a kind gift of Dr Jean Rivier, Sentia Medical Sciences, Salk Institute for Biological Studies.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication July 18, 2017; revised August 3, 2018; accepted for publication August 17, 2018.

Available online November 12, 2018.

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0091-6749/\$36.00

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<https://doi.org/10.1016/j.jaci.2018.08.053>

releasing factor receptor subtype 1 (CRF₁) expressed on MCs.

Objective: In this study we investigated the role of corticotropin-releasing factor receptor subtype 2 (CRF₂) as a modulator of stress-induced MC degranulation and associated disease pathophysiology.

Methods: *In vitro* MC degranulation assays were performed with bone marrow–derived mast cells (BMMCs) derived from wild-type (WT) and CRF₂-deficient (CRF₂^{−/−}) mice and RBL-2H3 MCs transfected with CRF₂-overexpressing plasmid or CRF₂ small interfering RNA. *In vivo* MC responses and associated pathophysiology in IgE-mediated passive systemic anaphylaxis and acute psychological restraint stress were measured in WT, CRF₂^{−/−}, and MC-deficient *Kit*^{W-sh/W-sh} knock-in mice.

Results: Compared with WT mice, CRF₂^{−/−} mice exhibited greater serum histamine levels and exacerbated IgE-mediated anaphylaxis and colonic permeability. In addition, CRF₂^{−/−} mice exhibited increased serum histamine levels and colonic permeability after acute restraint stress. Experiments with BMMCs and RBL-2H3 MCs demonstrated that CRF₂ expressed on MCs suppresses store-operated Ca²⁺ entry signaling and MC degranulation induced by diverse MC stimuli. Experiments with MC-deficient *Kit*^{W-sh/W-sh} mice systemically engrafted with WT and CRF₂^{−/−} BMMCs demonstrated the functional importance of MC CRF₂ in modulating stress-induced pathophysiology.

Conclusions: MC CRF₂ is a negative global modulator of stimuli-induced MC degranulation and limits the severity of IgE-mediated anaphylaxis and stress-related disease pathogenesis. (J Allergy Clin Immunol 2019;143:1865-77.)

Key words: Mast cell, corticotropin-releasing factor receptor, stress, anaphylaxis, allergy, intestinal permeability

Psychological stress has a major influence on immune function and is a risk factor in many immune-related disorders. Although numerous studies have investigated the influence of different stress paradigms on immune function and disease susceptibility, the underlying signaling pathways remain to be elucidated. Mast cells (MCs) have become recognized as important early immune effector cells in the stress response and stress-related pathophysiology. MCs are strategically positioned close to neurovascular units and host-environment interfaces (eg, mucosal epithelial barriers) and express a repertoire of receptors to sense and respond rapidly to a continuous array of stress signals from neuroendocrine, immunologic, and environmental stimuli. When activated, MCs release an array of preformed granule mediators, including histamine proteases, and select cytokines through degranulation, which trigger rapid and robust physiologic effects, such as changes in blood flow, increased endothelial and epithelial permeability, hypersecretion, and immune cell activation and recruitment.¹⁻⁶ Stress-induced MC degranulation and the associated tissue pathophysiology likely represent a critical host defense strategy to mobilize critical resources for the fight or flight response and to enhance immune function; however, if uncontrolled MC activation can be detrimental and has been linked to the onset and severity of diseases, including allergy, asthma, and irritable bowel syndrome (IBS).⁷⁻¹¹

The corticotropin-releasing factor (CRF) system is a major stress regulatory system in the body¹² and is composed of a family of peptide-related CRFs and the related family of urocortins

Abbreviations used

BMMC:	Bone marrow–derived mast cell
CRF:	Corticotropin-releasing factor
CRF ₁ :	Corticotropin-releasing factor subtype 1
CRF ₂ :	Corticotropin-releasing factor subtype 2
DNP:	Dinitrophenyl
ER:	Endoplasmic reticulum
GFP:	Green fluorescent protein
GPCR:	G protein–coupled receptor
β-Hex:	β-Hexosaminidase
MC:	Mast cell
PD:	Potential difference
PSA:	Passive systemic anaphylaxis
shRNA:	Short hairpin RNA
siRNA:	Small interfering RNA
SOCE:	Store-operated Ca ²⁺ entry
STIM1:	Stromal interacting molecule 1
TBS-T:	Tris-buffered saline with 0.1% Tween 20
TER:	Transepithelial electrical resistance
TRPC1:	Transient receptor potential canonical 1
WT:	Wild-type

(I-III), which mediate their actions through the G protein–coupled receptors (GPCRs) CRF₁ and CRF₂. The role of the CRF system has been extensively studied in the central nervous system with regard to HPA axis regulation and neurobehavioral paradigms.^{12,13} More recently, the CRF system has been shown to be highly active in peripheral immunologic and infectious challenge conditions,¹⁴⁻¹⁷ suggesting an important role in immune function. Comparatively little is known about the role of the CRF system in regulating the function of specific immune cells, such as MCs. MCs express both CRF₁ and CRF₂¹⁸⁻²⁰ and can synthesize and release ligands CRF and urocortins.^{21,22} Pharmacologic stimulation of CRF receptors on human and rodent MCs *in vitro* was shown to induce canonical GPCR signaling pathways, such as cyclic AMP Ca²⁺ and phosphorylated extracellular signal-regulated kinase, and the selective release of *de novo* synthesized growth factors and cytokines,^{18,19,23} whereas CRF receptor ligands did not induce degranulation. The *in vivo* role of MC-expressed CRF receptors has remained elusive; however, we recently demonstrated that CRF₁ expressed on MCs acts as a positive modulator of stress-induced MC degranulation and associated tissue pathophysiology in response to anaphylaxis and psychological stress.²²

In the present study we demonstrate that the complementary CRF receptor subtype CRF₂ is a negative modulator of MC degranulation and associated pathophysiologic responses to immunologic and psychological stressors, thus further supporting a critical homeostatic role for the MC-specific CRF system in MC activation and MC-associated diseases.

METHODS

Ethics statement

All protocols were approved by the North Carolina State University (protocol 09-047B) and Michigan State University's Institutional Animal Care and Use Committee (protocol 03/15-039-00).

Animals

Founding breeders for all mice strains were obtained from the Jackson Laboratory (Bar Harbor, Me) and housed in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols. C57BL/6 (stock no. 000664), and *Ki^{W-sh/W-sh}* mice (stock no. 012861) used in this study were derived from homozygous breeders. Heterozygous *CRF₂^{+/-}* mice (B6; 129-*Crhr2^{tm1Jsp}/J*; stock no. 010842) were purchased and bred to obtain wild-type (WT) *CRF₂^{+/+}* and *CRF₂^{-/-}* (knockout) mice. In the knockout mice the 3 exons which encode for the intracellular loop of the *CRF₂* cytoplasmic region have been replaced with a neomycin resistance cassette, as described earlier.²⁴ Standard PCR was used to genotype WT and *CRF₂^{-/-}* mice and bone marrow-derived mast cells (BMMCs) through both in house and commercial services (GeneTyper, New York, NY) by using protocols published at www.jax.org.

Passive systemic anaphylaxis

WT and *CRF₂^{-/-}* female mice (8 weeks of age) were sensitized by means of intraperitoneal injection with 20 μ g of IgE monoclonal anti-dinitrophenyl in 100 μ L of PBS. The following day, mice were challenged with 1 mg of dinitrophenyl (DNP) in 100 μ L of PBS (administered intraperitoneally). The mice were constantly observed, and rectal body temperature was recorded for 0, 15, 30, 60 and 120 minutes after DNP challenge (TH-5 Thermalert; Physitemp, Clifton, NJ). Mice were euthanized at 0 and 30 minutes after DNP injection for serum collection through cardiac puncture and stored at -80°C until serum histamine levels were quantified by using a commercial EIA kit (Oxford Biomedical Research, Rochester Hills, Mich).

For antalarmin experiments, passive systemic anaphylaxis (PSA) was performed as indicated above with the additional treatment group of antalarmin-treated *CRF₂^{-/-}* mice. Antalarmin (15 mg/kg) was injected (intraperitoneally) in male mice 15 minutes before DNP challenge. Mice were euthanized 30 minutes after DNP injection, and plasma was collected by means of cardiac puncture for later histamine analysis.

Histamine measurements

Histamine concentrations were quantified in serum, plasma, and cell pellets and supernatants from stimulated MC cultures with a histamine EIA kit (Oxford Biomedical Research).

Quantification of tissue MC numbers

Small intestinal mesentery windows from WT and *CRF₂^{-/-}* mice were whole mounted on glass slides, fixed with Carnoy fixative, and stained with toluidine blue (Sigma-Aldrich, St Louis, Mo), as described previously.²⁵ Hearts and ears from the same mice were fixed in 4% paraformaldehyde embedded in paraffin, and deparaffinized sections were stained with toluidine blue. Toluidine blue-stained MCs were counted in 5 nonoverlapping microscopic fields at a magnification of $\times 400$. Each field contained tissue sections that filled the entire high-power field. Mesentery MCs were counted in at least 6 mesenteric windows per mouse ($n = 4$ per genotype). Before performing tissue counts, each slide was coded so that counts were performed in a blinded manner. The average number of MCs per high-power field was then calculated for each tissue and within each genotype.

WBC differential count

Blood was collected by means of cardiac puncture from WT and *CRF₂^{-/-}* female mice and placed into EDTA-treated tubes (Microvette, Nümbrecht, Germany). Blood smears were performed, followed by a differential WBC count (number of basophils/100 WBCs).

Ussing chamber studies

Distal small intestine (ileum) was harvested from each mouse immediately after death and opened along the antimesenteric border. The intestinal mucosa was stripped from the seromuscular layer in oxygenated (95% O_2 and 5%

CO_2) Ringer solution (154 mmol/L Na^+ , 6.3 mmol/L K^+ , 137 mmol/L Cl^- , 0.3 mmol/L H_2PO_4 , 1.2 mmol/L Ca^{2+} , 0.7 mmol/L Mg^{2+} , and 24 mmol/L HCO_3^- [pH 7.4]) and mounted in 1.13-cm² aperture Ussing chambers (World Precision Instruments, Sarasota, Fla). Ileal mucosa was bathed on the serosal and mucosal sides with 10 mL of Ringer solution. The serosal bathing solution contained 10 mmol/L glucose, which was osmotically balanced on the mucosal side with 10 mmol/L mannitol. Bathing solutions were oxygenated (95% O_2 and 5% CO_2) and circulated in water-jacketed reservoirs maintained at 37°C .

The spontaneous potential difference (PD) was measured by using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes by using a voltage clamp that corrected for fluid resistance. Tissues were maintained in the short-circuited state, except for brief intervals to record the open-circuit PD.

Transepithelial electrical resistance (TER; measured as $\Omega \cdot \text{cm}^2$) was calculated from the spontaneous PD and short-circuit current, as previously described.²⁶ After a 30-minute equilibration period on the Ussing chambers, TER was recorded at 15-minute intervals over a 1-hour period and then averaged to derive the basal TER values for a given animal. After a 30-minute equilibration period on Ussing chambers, FD4 (100 mg/mL; Sigma, St Louis, Mo) was added to the mucosal bathing reservoir of the Ussing chambers. After a 15-minute equilibration period, standards were taken from the serosal side of each chamber, and a 60-minute flux period was established by taking 0.5-mL samples from the mucosal compartment. The quantity of FD4 was established by measuring the fluorescence in mucosal reservoir fluid samples in a fluorescence plate reader at 540 nm. Data are presented as the rate of FD4 flux in nanograms of FD4 per minute per square centimeter.

BMMC and RBL-2H3 cell cultures

Bone marrow cells were derived from female WT and *CRF₂^{-/-}* mice at approximately 4 to 6 weeks of age. Isolated bone marrow progenitor cells were cultured in RPMI 1640 media (with L-glutamine) supplemented with FBS (10%), sodium pyruvate (1 mmol/L), MEM nonessential amino acids (1 $\times$), HEPES buffer (10 mmol/L), penicillin (100 U/mL) and streptomycin (100 μ g/mL), and recombinant cytokines (stem cell factor, 5 ng/mL; IL-3, 5 ng/mL). After 4 weeks, cultures were composed mainly of MCs (93%), as determined by using toluidine blue staining and flow cytometric analysis (BD LSR II) by using fluorescently labeled c-kit (BioLegend, San Diego, Calif), high-affinity IgE receptor (Fc ϵ RI) antibodies (eBioscience, San Diego, Calif), and *CRF₂* anti-rabbit. Media and supplements were purchased from Cellgro (Corning, Manassas, Va) and recombinant cytokines from R&D Systems (Minneapolis, Minn). The rat basophilic leukemia cell MC line (RBL-2H3) was grown in MEM medium with 10% FBS.

Confocal immunofluorescence staining

BMMCs and peritoneal MCs were obtained from 8-week-old female C57BL/6 mice, as previously described.²⁵ Cells were pelleted, washed once with 1 $\times$ PBS, cytospun onto slides, and fixed with 4% paraformaldehyde for 20 minutes at 4°C . Slides were blocked at room temperature for 1 hour with blocking buffer (10% normal donkey serum and 0.3% Triton X-100) in 1 $\times$ PBS, permeabilized (0.1% Triton X-100), and incubated with goat anti-*CRF₁* (NB1-00175; Novus Biologicals, Centennial, Colo) and rabbit anti-*CRF₂* (NBP100-56485, Novus Biologicals) in dilution buffer at a concentration of 1:100 diluted in 1% BSA in a humidified chamber overnight at 4°C . Slides were washed 3 times for 5 minutes and then incubated with anti-rabbit-Cy3 and anti-goat-fluorescein isothiocyanate secondary antibodies in dilution buffer (1:300) for 1 hour at room temperature in the dark. Slides were washed 3 times for 5 minutes with 1 $\times$ PBS, and Pro-Long Diamond Antifade Mountant with 4'-6-diamidino-2-phenylindole dihydrochloride (P36971; Thermo Fisher, Waltham, Mass) was added before placement under cover slips. Confocal images were obtained with the Olympus FV 1000 confocal laser scanning microscope (Olympus America, Center Valley, Pa).

β -Hexosaminidase assay

RBL-2H3 cells or murine BMMCs (2×10^6 cells/mL) were sensitized overnight at 37°C with 1 μ g/mL anti-DNP IgE (Sigma-Aldrich) in complete RPMI medium with cytokines. The following day, cells were washed and resuspended in Tyrode buffer, and BMMCs were seeded in a 96-well plate (0.45×10^6 cells/well). Cells were equilibrated for an hour and stimulated with the indicated concentrations of DNP-albumin (Sigma-Aldrich) for 1 hour at 37°C. For A23187 and C48/80 stimulation, unsensitized BMMCs were stimulated for 1 hour. β -Hexosaminidase (β -Hex) activity was measured by addition of *p*-nitrophenol-N-acetyl- α -D-glucosaminide in 0.1 mol/L sodium citrate (pH 4.5) for 1 hour at 37°C. The reaction was terminated with 0.1 mol/L carbonate buffer (pH 10.0), and absorbance was recorded at 405 nm. The percentage of degranulation was calculated by dividing optical absorbance of the supernatant by the sum of optical absorbance of the supernatant and cell pellet (lysed with 0.1% Triton X-100) and multiplying by 100.

CRF₂ overexpression and knockdown in RBL-2H3 MCs

The Lenti-ORF clone of CRF₂ (*CRHR2*), transcript variant 1, and membrane bound green fluorescent protein-tagged plasmid were obtained from OriGene (Rockville, Md; catalog no. RG222881). CRF₂ short hairpin RNA (shRNA) clones (MSH028936-LVRU6MH) were purchased from GeneCopoeia (Rockville, Md). DH5 α -competent cells were obtained from Invitrogen (Carlsbad, Calif; catalog no. 18265017), and the plasmids were transfected and plated on selective antibiotic resistance LB plates, according to the manufacturer's protocol. After overnight incubation at 37°C, a single colony was picked for each clone and inoculated on 2.5 mL of LB medium containing antibiotics and kept for 8 hours at 37°C at 200 rpm. A total of 600 μ L of culture inoculum was transferred into 300 mL of LB broth on a sterile 1-L flask with antibiotics and kept at 200 rpm at 37°C overnight. Plasmid was extracted, and the concentration was measured according to the manufacturer's protocol (catalog no. 12662; Qiagen, Hilden, Germany).

Human embryonic kidney cell 293Ta lentivirus cells were seeded at a concentration of 1.5×10^6 cells per well (6 well), and 2.5 μ g of CRF₂-overexpressed or CRF₂ shRNA, along with respective control empty green fluorescent protein (GFP) or scrambled shRNA plasmid control DNA, was transfected with the Lentiviral Packaging Kit (catalog no. TR30022; OriGene), according to the manufacturer's protocol. After overnight incubation at 37°C with 5% CO₂, transfection medium was replaced with normal 5% Dulbecco modified Eagle medium with Polybrene (1 μ L of 4 mg/mL). Forty-eight hours after transfection, the supernatant was collected from the 6-well plate and filtered onto a Nalgene ultracentrifuge tube, and 5 mL of 20% sucrose was added to the bottom of the tube. After centrifugation at 25,000 rpm for 2 hours at 4°C, the supernatant was discarded, and the lentivirus-overexpressing CRF₂ plasmid or CRF₂ shRNA pellet was resuspended with 150 μ L of 4% lactose and kept on ice for 15 minutes and centrifuged at a high speed for 1 minute. Lentiviral aliquots were stored at -80°C until transfection.

Lentivirus titering was done, and RBL-2H3 cells at a concentration of 2.5×10^5 cells were plated on a 12-well plate and grown overnight. Lentivirus-overexpressing *CRHR2* or CRF₂ shRNA plasmid at a multiplicity of infection of 40 and Polybrene at a final concentration of 8 μ g/mL was added to each well, and plates were centrifuged at 300g for 60 minutes at 30°C. The plate was incubated at 37°C overnight, and the medium was replaced with 15% MEM. After 48 hours of transfection, cells were trypsinized and transferred into T75 flasks. The RBL-2H3 cells overexpressing CRF₂ or CRF₂ shRNA tagged with GFP (CRF₂ overexpressed) or mCherry (CRF₂ shRNA) were sorted on flow cytometry (FACSARIA II; Becton Dickinson, Franklin Lakes, NJ [UNC, Chapel Hill, NC]) based on the GFP- or mCherry-positive cells, expanded in culture, and the RBL-2H3 cell aliquots were stored in liquid nitrogen for further study.

CRF₂ small interfering RNA knockdown in RBL-2H3 cells

RBL-2H3 cells grown overnight seeded at a concentration on 5×10^5 in a 6-well plate were transfected with CRF₂ small interfering RNA (siRNA; NM-

022714; Sigma) or scrambled siRNA (SIC00-1; Sigma) at 150 ng by using Qiagen Hi-Perfect Transfection Reagent, according to the manufacturer's protocol. Six hours after transfection, normal growth medium was added to each well and incubated at 37°C with 5% CO₂ for 48 hours. After incubation, transfected cells were primed or not with IgE overnight and stimulated with 1 hour of DNP or A23187 (non-IgE primed), and the β -hex, histamine, and calcium mobilization assay was done, as described above.

Human MC experiments with LAD2 cells

LAD2 cells provided by A. Kirshenbaum (National Institutes of Health, Bethesda, Md) were cultured in StemPro-34 (Life Technologies, Carlsbad, Calif) medium supplemented with StemPro-34 Nutrient Supplement (Life Technologies) L-glutamine (2 mmol/L; Life Technologies), penicillin (100 U/mL)/streptomycin (100 μ g/mL), and recombinant human stem cell factor (100 ng/mL; PeproTech, Rocky Hill, NJ). Cell density was kept at less than 0.5×10^6 /mL, and half the amount of medium was replaced weekly. Cells were sensitized overnight with 100 ng/mL biotinylated human IgE (US Biological, Salem, Mass). On the following day, cells were washed with Tyrode buffer to remove excess IgE and stimulated with vehicle (0.1% BSA in PBS), streptavidin (100 ng/mL), and 1 to 100 nmol/L human Astressin 2B (kind gift from Jean Rivier, Salk Institute, La Jolla, Calif). β -Hex release was measured after 1 hour of stimulation, as described above.

RT-PCR

Total RNA was extracted from heart tissue and BMMCs by using TRIzol Reagent (Invitrogen), followed by DNase treatment and purification with the PureLink RNA mini kit (Life Technologies). cDNA was synthesized and amplified by using the SuperScript III One-Step RT-PCR System with CRF₁-specific primers (KSPQ12012 Sigma) and CRF₂ specific primers (KSPQ12012, Sigma). GAPDH forward 5'-TGCACCACCAACTGCTTAG-3' and reverse 5'-GGATGCAGGGATGATGATGTTTC-3' was used as normalizing control. For RBL-2H3 cells, total RNA was isolated by using the Qiagen RNeasy kit and transcribed to cDNA by using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). CRF₂ expression was determined by using CRF₂-specific primers (forward Rat 1_Chr2: ACTCTACTATGA-GAATGAGCAG and reverse Rat 1_Chr2: TGACGATGTTGAACA-GAAAC; Sigma-Aldrich).

Intracellular Ca²⁺ measurements

RBL-2H3 cells and murine BMMCs derived from WT and CRF₂^{-/-} mice were used for this study. On the day of the experiment, cells were washed and resuspended in calcium assay buffer. Changes in intracellular calcium levels in response to stimulus were detected by using the Fluo 4 NW Calcium assay kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Briefly, cells were loaded at 37°C for 30 minutes with the fluorescent Ca²⁺ indicator Fluo 4 NW in the presence of probenecid, followed by incubation at room temperature for another 30 minutes. Changes in fluorescence were measured by stimulating the cells by adding different concentrations of DNP/HSA, A23187, or C48/80. Change in fluorescence was measured by using the FDSS/ μ CELL kinetic plate reader (Hamamatsu, Hamamatsu, Japan) or Fluoroskan Ascent FL microplate reader (Thermo Fisher Scientific) at 480-nm excitation and 540-nm emission. The ratio was calculated based on the average increase in fluorescence divided by the baseline and determined for each time point after the addition of stimulus.

Store-operated Ca²⁺ entry channel expression

BMMC lysates were prepared by resuspending cells in RIPA buffer (Thermo Fisher Scientific) containing protease and phosphatase inhibitors (Thermo Fisher Scientific) at 4°C. Lysates were sonicated and centrifuged at 21,000g for 10 minutes. The protein concentration was measured by using the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, Calif). Cellular proteins were separated by means of SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% wt/vol BSA in

Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 hour at room temperature, washed in TBS-T, and incubated with the rabbit anti-human antibodies anti-stromal interacting molecule 1 (STIM1; #ACC-063; 1:500 dilution; Alomone Labs, Jerusalem, Israel), anti-transient receptor potential canonical 1 (TRPC1; #ACC-010; 1:600 dilution; Alomone Labs), anti-Orai1 (#ACC-060; 1:400 dilution; Alomone Labs), and β -actin (4970, 1:1000; Cell Signaling, Danvers, Mass) diluted in 5% wt/vol BSA in TBS-T overnight at 4°C or 2 hours at room temperature and then washed with TBS-T. Subsequently, the membranes were incubated with rabbit polyclonal secondary antibody (7074; 1:2000; Cell Signaling) for 1 hour at room temperature, washed with TBS-T, and incubated in SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Bands were visualized with the ChemiDoc MP Imager (Bio-Rad Laboratories), and Image Lab software (version 4.1) was used for densitometric analysis.

Restraint stress model

Female mice were placed in individual, transparent, 50-mL, plastic conical tubes modified with air holes for a 3-hour period. Control mice remained in their original home cages for 3 hours without food and water to avoid the confounding effects of water or food intake during the 3-hour experimental period. After restraint stress, mice were immediately euthanized by means of CO₂ inhalation, and serum and ileal mucosa were collected for measurement of serum histamine and intestinal permeability, respectively.

BMMC engraftment in *Kit^{W-sh/W-sh}* mice

Female *Kit^{W-sh/W-sh}* mice (8–10 weeks of age) were injected intraperitoneally with 1×10^7 BMMCs (suspended in 200 μ L of sterile $1 \times$ PBS) derived from CRF₂^{+/+} (WT) or CRF₂^{-/-} mice. At 16 weeks of age (4 weeks after engraftment), engrafted mice were euthanized by means of CO₂ inhalation. Intestinal mesentery window sections were harvested, fixed in Carnoy's fixative, and stained with toluidine blue to confirm engraftment of BMMCs.

Statistical analysis

In vivo and *in vitro* studies are presented as means $\pm$ SEs from a representative experiment with 6 to 8 animals per treatment group (unless otherwise specified). Experimental results were repeated in a minimum of 2 independent experiments, whereas some experiments were repeated in greater than 2 independent experiments (noted in the figure legends). Comparisons between multiple experimental groups were performed by using a 1- or 2-way ANOVA where appropriate with a Tukey posttest. Comparisons between 2 treatments were analyzed by using an unpaired 2-tailed *t* test. *P* values of less than .05 were considered statistically significant. All statistical analyses and calculations were performed with GraphPad Prism 5 software (GraphPad, La Jolla, Calif).

RESULTS

CRF₂ deficiency exacerbates PSA-induced MC degranulation, anaphylaxis, and intestinal permeability

To define the role of CRF₂ in MC-associated disease *in vivo*, we compared MC responses and associated pathophysiology in an MC-dependent model of IgE-mediated PSA. Compared with WT mice, CRF₂^{-/-} mice exhibited greater (by 4.1-fold) serum histamine levels measured at 30 minutes after PSA challenge (5017 ± 919 and 1208 ± 235 ng/mL in CRF₂^{-/-} and WT mice, respectively; *P* < .001; Fig 1, A). In agreement with serum histamine levels, CRF₂^{-/-} mice exhibited a greater peak reduction in body temperature measured at 30 minutes post-DNP challenge ($\Delta -4.55^\circ\text{C} \pm 0.85^\circ\text{C}$ and $\Delta -6.8^\circ\text{C} \pm 0.54^\circ\text{C}$ in WT and CRF₂^{-/-} mice, respectively; *P* = .04; Fig 1, B).

Measurement of cardiac blood volume reduction, an index of systemic vasodilation and fluid extravasation induced by

PSA,^{27,28} was also greater in CRF₂^{-/-} mice at 30 and 120 minutes after DNP challenge compared with that in WT mice (Fig 1, C), thus indicating a more severe hypovolemic shock response in CRF₂^{-/-} mice. Intestinal permeability, measured as FD4 flux rate and reduced TER across ileal mucosa mounted on Ussing chambers, was greater in CRF₂^{-/-} mice compared with that in WT mice 120 minutes after PSA (Fig 1, D and E). Quantification of tissue MCs by using toluidine blue staining of heart, intestinal mesentery, and skin showed that WT and CRF₂^{-/-} mice had similar distributions and numbers of tissue MCs (Fig 1, F–H). Furthermore, circulating basophil numbers were similar between WT and CRF₂^{-/-} mice, confirming that increased histamine levels in CRF₂^{-/-} mice were not due to increased basophil numbers.

Given that many MC-dependent diseases exhibit a female sex bias and based on our previous studies demonstrating sex differences in MC responses,²⁵ we also compared the influence of CRF₂ deficiency on PSA-induced serum histamine responses in female and male mice. These studies confirmed our previous research showing that female mice exhibit a greater release of serum histamine compared with male mice. CRF₂^{-/-} deficiency exacerbated serum histamine responses in both female and male mice (see Fig E1 in this article's Online Repository at www.jacionline.org). Therefore we conducted subsequent experiments predominantly in female mice. Together, these experiments revealed a significant *in vivo* role for CRF₂ in IgE-mediated MC degranulation, anaphylaxis, and intestinal permeability.

CRF₂ expressed on MCs is a negative modulator of MC degranulation

CRF receptors are widely expressed in numerous cell types, including MCs,^{20,29,30} but little is known regarding the localization or functional role of CRF₂ in MC function. By using confocal microscopy, CRF₂ was found to be localized to both the surface and intracellularly in BMMCs and peritoneal (tissue) MCs (Fig 2). Furthermore, a nuclear staining pattern of CRF₂ was also observed predominantly in BMMCs compared with peritoneal MCs. Flow cytometry confirmed the surface expression of CRF₂ in BMMCs (see Fig E2, G, in this article's Online Repository at www.jacionline.org). CRF₁ showed a different pattern of expression, with a predominant surface localization in peritoneal MCs and BMMCs, and some intracellular staining was observed mostly in BMMCs.

To explore the functional role of MC CRF₂^{-/-}, we conducted *in vitro* degranulation assays with BMMCs derived from WT and CRF₂^{-/-} mice. In line with *in vivo* PSA studies, CRF₂^{-/-} BMMCs exhibited exacerbated degranulation responses measured as enhanced β -hex release after IgE-Fc ϵ RI cross-linking (Fig 3, A). Transmission electron microscopy revealed a more activated appearance of CRF₂^{-/-} BMMCs after IgE/DNP compared with WT BMMCs, thus supporting the β -hex data (Fig 3, D).

To determine whether the exacerbated MC degranulation responses observed in CRF₂^{-/-} BMMCs were specific to IgE-Fc ϵ RI-mediated pathways, WT and CRF₂^{-/-} BMMCs were also stimulated with non-IgE-dependent MC stimuli, including C48/80, which acts through the GPCR Mrgpb2,³¹ and A23187, which acts on downstream store-operated Ca²⁺ entry (SOCE) mechanisms from intracellular (eg, endoplasmic reticulum [ER]) and extracellular stores.³² Similar to IgE stimulus,

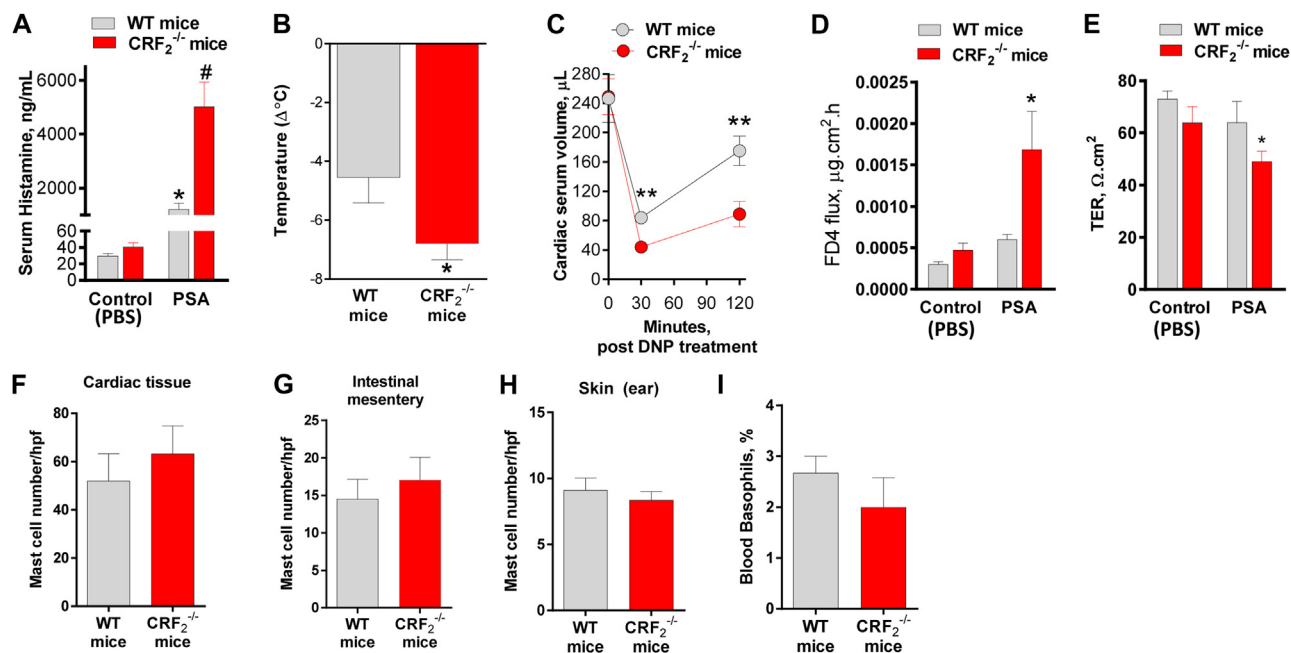


FIG 1. CRF₂ deficiency exacerbates PSA and intestinal permeability. WT (CRF₂^{+/+}) and CRF₂^{-/-} female mice (6-8 weeks of age) were sensitized systemically overnight with IgE and challenged with either PBS vehicle (control) or anti-IgE DNP to induce PSA. **A**, Serum histamine levels were measured 30 minutes after vehicle or DNP challenge. **B**, Rectal temperature was recorded at 0 and 30 minutes, and change in body temperature was calculated and presented as Δ degrees Celsius. **C**, Cardiac blood volume was measured at 0, 30, and 120 minutes after DNP challenge. **D** and **E**, One hundred twenty minutes after DNP challenge, *ex vivo* colonic permeability was measured on Ussing chambers as FD4 flux rate (Fig 1, **D**) and TER (Fig 1, **E**). **F-H**, Tissues from WT and CRF₂^{-/-} mice were fixed and stained with toluidine blue, and MC counts were performed and presented as MCs per high-power field (hpf). **G**, Blood basophils were counted in blood smears and expressed as the percentage of basophils per 100 WBCs. Data are means ± SEMs from a representative experiment with 6 animals per group. Experiments were repeated in 6 (serum histamine levels and body temperature) or 2 (cardiac volume and MC and basophil counts) independent replicates. #, *Symbols differ by *P* < .05 by using a 1-way ANOVA (Fig 1, **A** and **D**). Different asterisks indicate significance determined by using unpaired 2-tailed *t* tests (Fig 1, **B**, **C**, and **E**): **P* < .05 and ***P* < .01.

CRF₂^{-/-} BMMCs exhibited exacerbated β-hex release in response to both A23187 and C48/80 (Fig 3, **B** and **C**). Because our previous work demonstrated that CRF₁ potentiates stimuli-induced MC degranulation,²² we investigated whether enhanced degranulation in CRF₂^{-/-} BMMCs could be due to a reciprocal upregulation of CRF₁ expression. Quantitative PCR showed that compared with WT BMMCs, CRF₂ expression was downregulated in CRF₂^{-/-} BMMCs (by approximately 40%) but CRF₁ expression was not significantly altered (see Fig E3, **A** and **B**, in this article's Online Repository at www.jacionline.org). It should be noted that because the loss of CRF₂ activity in CRF₂^{-/-} BMMCs (derived from CRF₂^{-/-} mice) is a result of a functional deletion of a C-terminal region, CRF₂ is still expressed, as shown in Fig E3, **A**, although at a lower level than WT BMMCs. There were no significant differences between WT and CRF₂^{-/-} BMMCs with regard to the cellular content of β-hex or phenotype determined based on c-kit/FcεRI expression by means of flow cytometric analysis (see Fig E2), confirming that enhanced degranulation in CRF₂^{-/-} BMMCs was not due to an increased intracellular granule mediator content or aberrant MC phenotype.

To further examine the link between CRF₂ expression on MCs and degranulation responses, we transfected CRF₂ plasmid into RBL-2H3 MCs to enhance CRF₂ expression. We confirmed the

specificity of CRF₂ overexpression by using quantitative PCR, which revealed upregulation of CRF₂ expression (by 6.4-fold, see Fig E3, **C**) compared with the empty GFP plasmid while having no significant effect on CRF₁ expression (see Fig E3, **D**). CRF₂-overexpressed RBL-2H3 MCs exhibited suppressed IgE/DNP-mediated β-hex release (by 47%) compared with empty plasmid controls (Fig 3, **E**). We also knocked down CRF₂ expression in RBL-2H3 MCs with CRF₂-directed siRNA, which resulted in a 2-fold increase in β-hex release after IgE/DNP stimulation, compared with scrambled siRNA controls (Fig 3, **F**). Pharmacologic inhibition of CRF₂ with the selective antagonist Astressin 2B enhanced IgE-FcεRI-induced β-hex release in both rat RBL-2H3 and human LAD2 MCs (Fig 4), thus confirming the genetic experiments and translational significance to human MCs.

Given our previous work showing that MC CRF₁ potentiates MC degranulation while in the present study CRF₂ suppressing MC degranulation, we hypothesized that MC CRF₁ and CRF₂ might function in concert to regulate or balance MC degranulation responses. To test this hypothesis, we pretreated CRF₂^{-/-} mice with the CRF₁ selective antagonist antalarmin, followed by induction of IgE-mediated PSA. Antalarmin significantly attenuated the exacerbated PSA-induced histamine release in CRF₂^{-/-} mice (see Fig E4 in this article's Online Repository at www.jacionline.org).

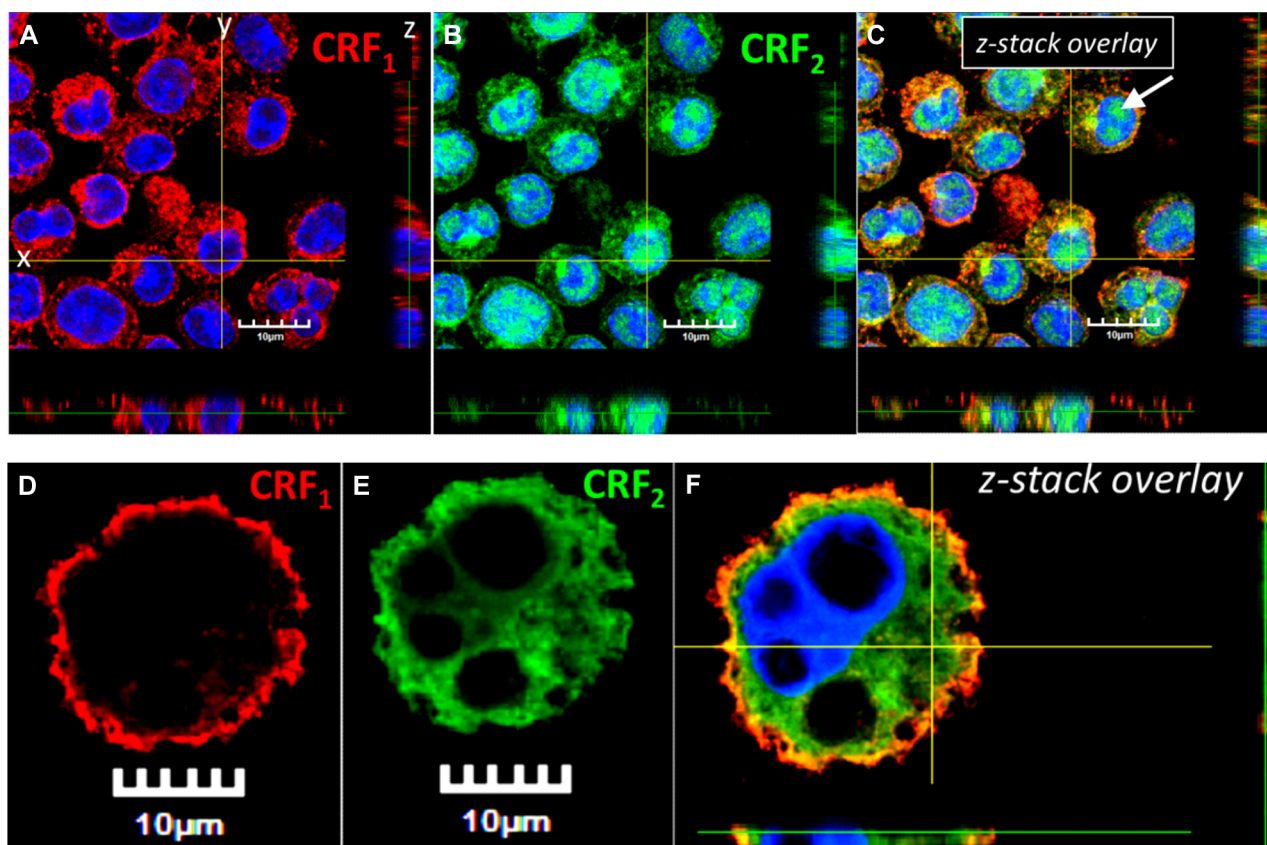


FIG 2. Localization of CRF₁ and CRF₂ in BMMCs and peritoneal MCs. CRF₁ (Cy3, red) and CRF₂ (fluorescein isothiocyanate [FITC], green) nuclei (4'-6-diamidino-2-phenylindole dihydrochloride [DAPI], blue) are shown. z-Stack overlay images show a single -plane cross-section through the horizontal plane (XY), sagittal plane (YZ), and coronal plane (XZ). **A-C**, Murine BMMCs. **D-F**, Peritoneal MCs. The white arrow indicates the nuclear staining pattern of CRF₂ (Fig 2, C).

CRF₂ negatively regulates SOCE in MCs

We next explored the mechanism by which CRF₂ caused suppression of stimuli-induced MC degranulation. Because CRF₂ was shown to negatively modulate MC degranulation induced by diverse MC stimuli that use upstream receptor-dependent pathway (IgE-FcεRI and C48/80) or downstream receptor-independent pathway (A23187) mechanisms, we hypothesized that CRF₂ was targeting a common downstream signaling mechanism used by MC degranulation stimuli. SOCE from intracellular (ER and Golgi) and extracellular stores is essential for MC granule exocytosis and degranulation.³³ Therefore we conducted Ca²⁺ mobilization experiments with WT and CRF₂^{-/-} BMMCs loaded with the Fluo4 Ca²⁺ indicator dye. These experiments showed that CRF₂^{-/-} BMMCs exhibited heightened IgE/DNP Ca²⁺ mobilization after IgE/DNP stimulation (Fig 5). Furthermore, CRF₂^{-/-} BMMCs also exhibited heightened Ca²⁺ signaling in response to other MC stimuli, including A23187, C48/80, and the muscarinic 3 receptor agonist carbachol (Fig 5), overall demonstrating the global inhibitory role of CRF₂ in response to diverse MC stimuli.

We next conducted similar experiments in Ca²⁺-replete or Ca²⁺-free conditions (1 mmol/L EDTA) to determine whether the heightened Ca²⁺ signaling in CRF₂^{-/-} BMMCs was due to enhanced release from intracellular stores, such as the ER, or through influx of extracellular Ca²⁺ through plasma membrane

channels. As anticipated, removal of extracellular Ca²⁺ with EDTA significantly reduced Ca²⁺ signals induced by IgE/DNP in both WT and CRF₂^{-/-} BMMCs (Fig 6, A and B). However, EDTA did not ablate the heightened Ca²⁺ signaling in CRF₂^{-/-} BMMCs compared with WT BMMCs (Fig 6, B and C), indicating heightened Ca²⁺ release from intracellular stores in CRF₂^{-/-} BMMCs. CRF₂^{-/-} BMMCs also exhibited increased expression of SOCE channels, including the ER Ca²⁺ sensor STIM1 and the plasma membrane channels TRPC1 and Orai1 (Fig 6, D-I). Together, these data indicate that CRF₂ is a negative and global modulator of stimuli-induced Ca²⁺ signaling with actions on intracellular release and subsequent extracellular Ca²⁺ entry.

CRF₂^{-/-} mice exhibit increased serum MC histamine and intestinal permeability in response to acute restraint stress

In vitro MC culture experiments revealed that MC CRF₂ exerted suppressive effects on MC degranulation induced by a diverse array of potent MC stimuli. Given these findings, we hypothesized that the global effects of CRF₂ on MC degranulation would extend to non-IgE-dependent *in vivo* models of MC degranulation. To test this hypothesis, we compared MC degranulation responses through measurement of serum histamine levels in WT and CRF₂^{-/-} mice after a 1-hour period of psychological

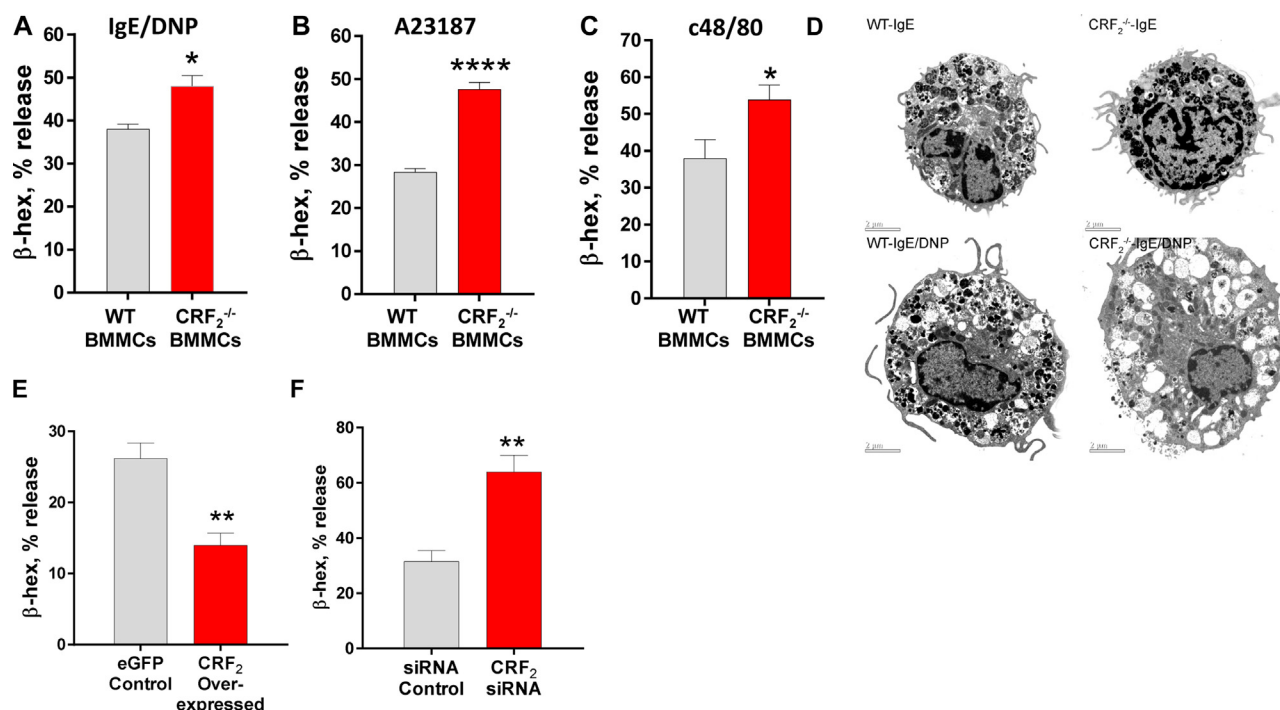


FIG 3. Genetic deficiency and overexpression of CRF₂ induces divergent effects on stimuli-induced MC degranulation. **A**, β -Hex percentage release from IgE-sensitized WT and CRF₂^{-/-} BMMCs stimulated with DNP (32 ng/mL). **B** and **C**, β -Hex release from WT and CRF₂^{-/-} BMMCs stimulated with A23187 (Fig 3, **B**) and C48/80 (Fig 3, **C**). **D**, Transmission electron microscopic analysis of WT and CRF₂^{-/-} BMMCs stimulated with IgE/DNP. **E** and **F**, β -Hex release from RBL-2H3 MCs transfected with CRF₂-overexpressing plasmid or empty GFP control or CRF₂ siRNA. Data are means $\pm$ SEMs from a representative experiment with 3 (bone marrow donors) per group. Experiments were repeated in 6 (Fig 3, **A-C**) or 2 (Fig 3, **E** and **F**) independent replicates. Significance between groups was determined by using an unpaired 2-tailed *t* test: **P* < .05, ***P* < .01, and *****P* < .0001.

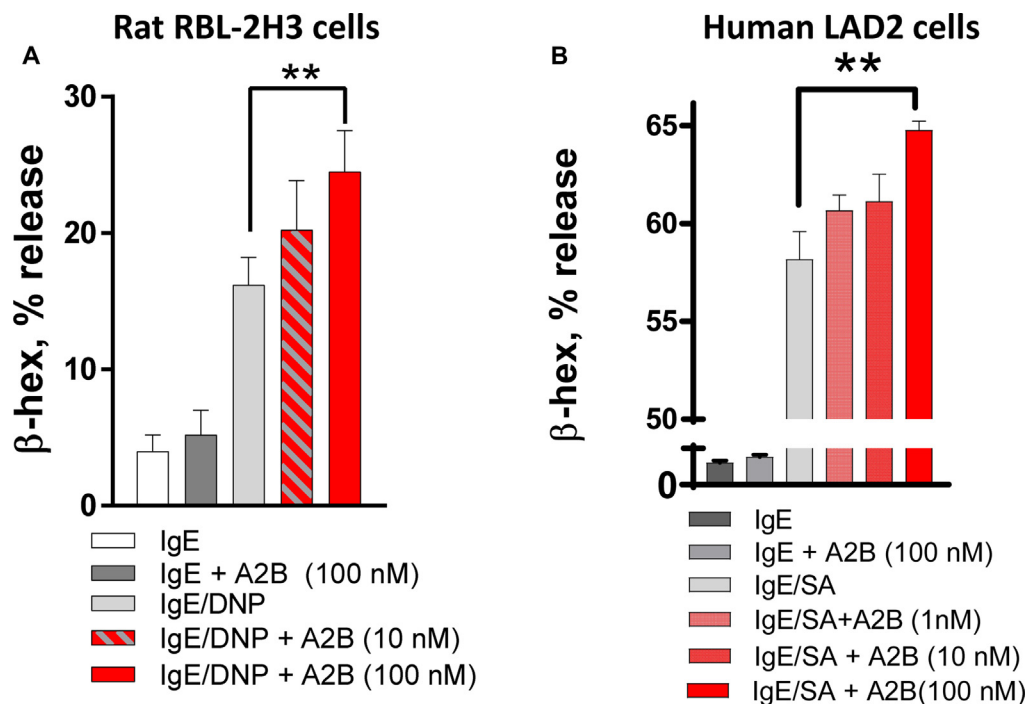


FIG 4. CRF₂ selective antagonism enhances MC degranulation in rodent and human MCs. β -Hex percentage release from RBL-2H3 MCs (**A**) and human LAD2 cells (**B**) after pretreatment with the CRF₂ antagonist drug Astrressin 2B (A2B) at indicated concentrations. ***P* < .01, 1-way ANOVA (*n* = 6 replicates per treatment). Experiments were repeated in 3 independent experiments.

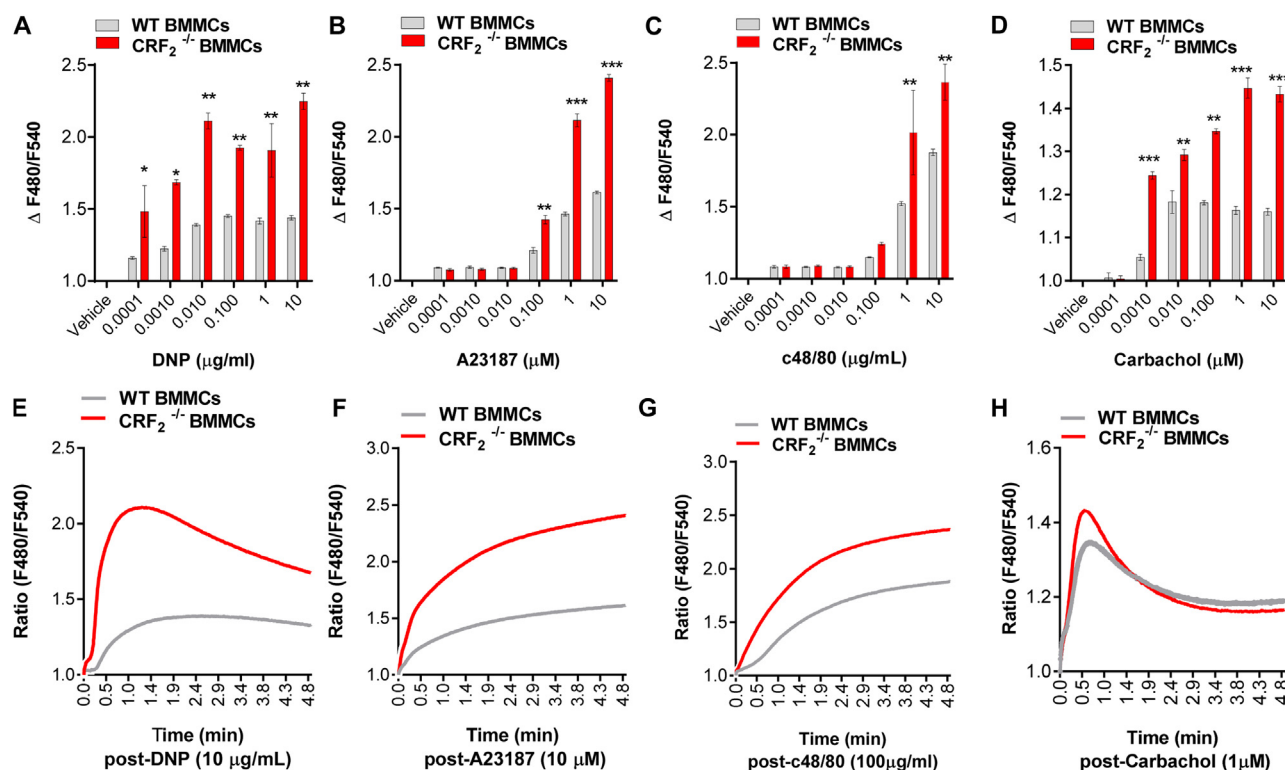


FIG 5. CRF₂^{-/-} BMMCs exhibit exacerbated Ca²⁺ mobilization responses to diverse MC stimuli. Murine BMMCs from female mice were loaded with Fluo 4 Ca²⁺ indicator and stimulated with indicated MC degranulation stimuli, as described in the Methods section. Intracellular Ca²⁺ mobilization (presented as ΔF480/F540 or representative traces) in response to indicated concentrations of IgE/DNP (A and E), A23187 (B and F), and C48/80 (C and G), and carbachol (D and H) is shown. Data shown are means ± SEs and are representative of 3 independent experiments performed in triplicates. Data were analyzed by using a 1-way ANOVA: **P* < .05, ***P* < .001, and ****P* < .0001.

restraint stress. Compared with WT mice, CRF₂^{-/-} mice exhibited higher serum histamine levels after restraint stress (97.7 ± 5.9 and 68.2 ± 7 ng/mL in CRF₂^{-/-} and WT mice, respectively; *P* < .01; Fig 7, A). We also assessed colonic permeability measured as FD4 flux rates on Ussing chambers, which showed a significantly increased FD4 flux rate in CRF₂^{-/-} mice after restraint stress compared with that seen in WT mice (Fig 7, B). Together, these data indicate that CRF₂ plays a global role in controlling MC degranulation and associated tissue pathophysiology induced by diverse stressors.

MC CRF₂ is a negative modulator of stress-induced intestinal permeability

The above experiments demonstrated an important role for CRF₂ as a negative modulator of MC-dependent intestinal permeability induced by PSA and restraint stress. Furthermore, *in vitro* experiments demonstrated the role of MC-specific CRF₂ in the control of stimuli-induced MC degranulation. We next determined the *in vivo* significance of CRF₂ expressed on MCs using a MC knock-in model. MC-deficient *Kit*^{W-sh/W-sh} mice were systemically engrafted with BMMCs derived from either WT or CRF₂^{-/-} mice. Twelve weeks after engraftment, mice underwent 3 hours of restraint stress, and colonic permeability was measured

in Ussing chambers. Compared with *Kit*^{W-sh/W-sh} mice engrafted with WT BMMCs, CRF₂^{-/-} engrafted mice exhibited the greater colonic permeability responses measured as FD4 flux rates (Fig 8, A). WT and CRF₂^{-/-} mice exhibited similar reductions in colonic TER after restraint stress (Fig 8, B). Toluidine blue staining and MC counts in intestinal mesenteric tissue confirmed an equal engraftment rate between WT and CRF₂^{-/-} BMMCs in MC-deficient *Kit*^{W-sh/W-sh} mice (Fig 8, C).

DISCUSSION

Stress is an important risk factor in the clinical onset and severity of important immunologic disorders, including allergy, asthma, and functional and inflammatory gastrointestinal disorders. MCs play a central role in many immunologic diseases and are a critical immune effector cell in the stress response. MCs are rapidly activated within minutes of exposure to diverse stressors, including psychological, chemical, and physical stressors, resulting in release of preformed granule mediators (eg, histamine, proteases, and TNF) through degranulation. Released MC mediators evoke profound physiologic effects in local and systemic tissues, such as increased epithelial and endothelial permeability, neuronal activation, and circulatory changes. Although stress-induced MC activation likely represents an early

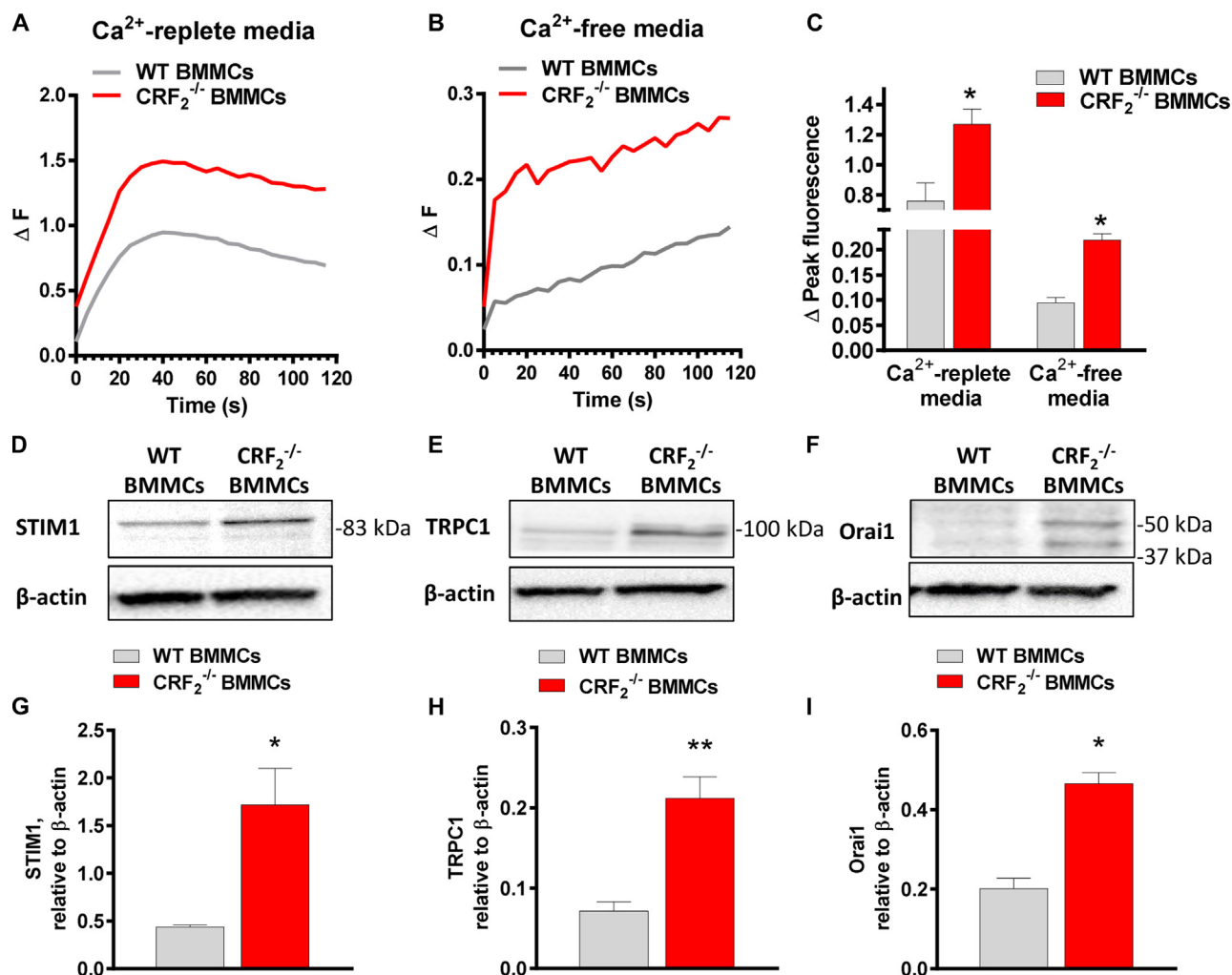


FIG 6. CRF₂^{-/-} BMMCs exhibit heightened intracellular Ca²⁺ store release and expression of SOCE channels. BMMCs derived from WT and CRF₂^{-/-} mice were loaded with Fluo 4, and intracellular Ca²⁺ levels were measured after stimulation with IgE/DNP. **A** and **B**, Representative intracellular Ca²⁺ traces for experiments conducted under Ca²⁺-replete (Fig 6, **A**) or Ca²⁺-free (1 mmol/L EDTA; Fig 6, **B**) conditions. **C**, Mean peak change in fluorescence after IgE/DNP stimulus presented as Δ peak fluorescence. **D-I**, Representative Western blots and densitometric analysis for STIM1 (Fig 6, **D** and **G**), TRPC1 (Fig 6, **E** and **H**), and Orai1 (Fig 6, **F** and **I**) in WT and CRF₂^{-/-} BMMCs. *Significance between groups was determined by using an unpaired 2-tailed t test (Fig 6, **C** and **G-I**). **P* < .05 and ***P* < .01.

defense or alarm mechanism to mobilize resources necessary for the subject to respond appropriately to the stressor, excessive or prolonged MC activation is harmful, leading to immune dysregulation and tissue damage and exacerbation of disease. Although the major MC stimuli have been identified, how environmental factors, such as stress, play a role in exacerbating or limiting MC activation remains poorly understood. Here we demonstrated a novel function for CRF₂ expressed on MCs as a negative modulator of stimuli-induced MC degranulation and MC-dependent pathophysiology associated with anaphylaxis and acute psychological stress.

Previous studies conducted *in vitro* with human and rodent MCs demonstrated that MCs express both CRF₁ and CRF₂ receptors^{22,23} and that CRF receptor ligands can induce canonical GPCR pathways, including cyclic AMP and phosphorylated extracellular signal-regulated kinase signaling.^{19,22,34} However, the precise role of the MC CRF system modulating MC function

and MC-mediated disease pathogenesis has remained elusive. Recently, we demonstrated that CRF₁ receptor signaling in the MC is a potentiator of stimuli-induced MC degranulation and intestinal pathophysiology induced by PSA and psychological restraint stress.³⁵ In the present study our data revealed that, in contrast to CRF₁, CRF₂ exerts inhibitory actions on MC degranulation because loss of CRF₂ activity in MCs through genetic or pharmacologic approaches resulted in enhanced stimuli-induced MC degranulation and exacerbated responses to PSA and acute restraint stress. Selective inhibition of CRF₁ was shown to reduce PSA-induced MC responses in CRF₂^{-/-} mice to levels seen in WT mice, overall indicating that MC CRF₁ and CRF₂ act in concert to upregulate and downregulate, respectively, the tone of MC degranulation and resultant MC-dependent pathophysiology. Therefore it is plausible that a disruption of MC CRF₁ and CRF₂ homeostasis could shift the balance between hypoactivated and hyperactivated MC states and ultimately disease activity.

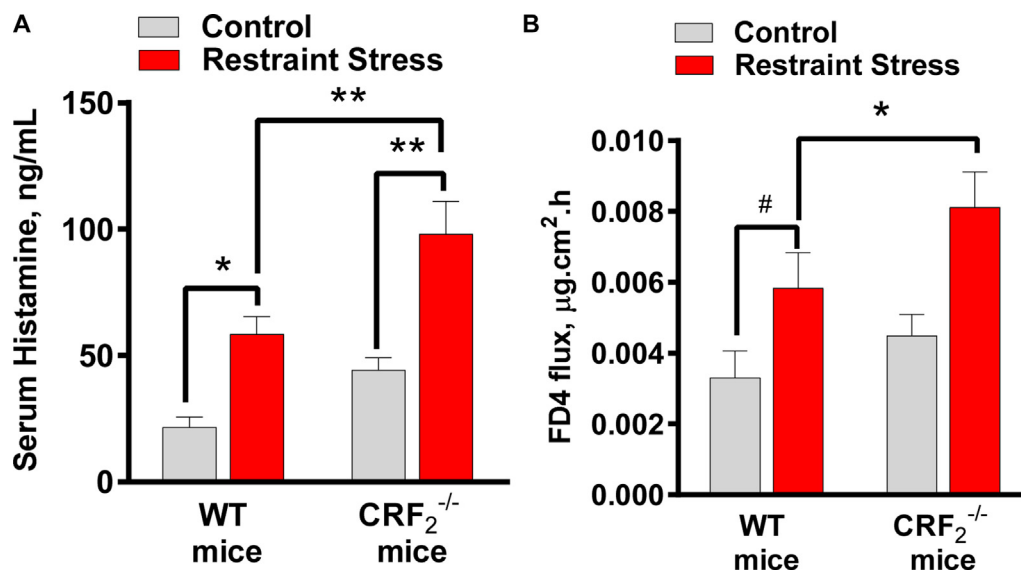


FIG 7. CRF₂^{-/-} mice exhibit heightened serum histamine levels and intestinal permeability after acute restraint stress. WT and CRF₂^{-/-} female mice (8-10 weeks of age) underwent 1 hour of restraint stress or housed under normal cage conditions (control), as described in the Methods section. Serum histamine levels (**A**) and colonic permeability (**B**) were measured by means of ELISA after 1 hour of restraint stress. Data are means $\pm$ SEMs (n = 8 mice per group). Data were analyzed by using 2-way ANOVA: **P* < .05, ***P* < .01, and #*P* = .07.

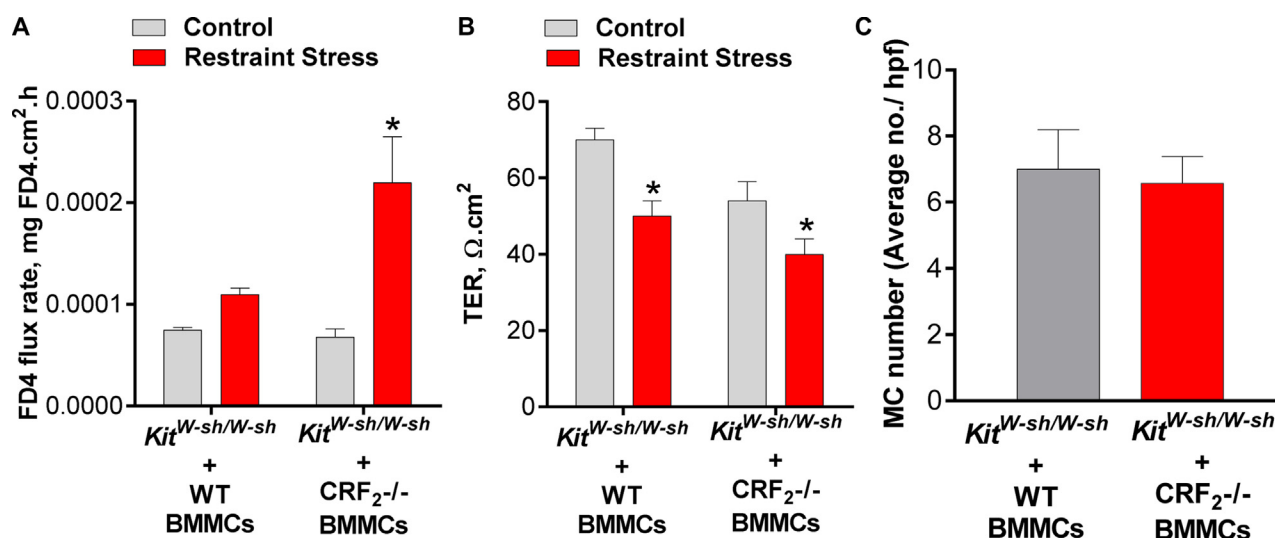


FIG 8. CRF₂ expressed on MCs is a critical modulator of stress-induced intestinal permeability. MC-deficient *Kit*^{W-sh/W-sh} mice were engrafted with BMMCs derived from WT or CRF₂^{-/-} mice. Twelve weeks after engraftment, mice were exposed to restraint stress for 3 hours. **A** and **B**, FD4 flux rate (Fig 8, A) and TER (Fig 8, B) measured from colon tissue mounted on Ussing chambers from WT and CRF₂^{-/-} BMMC-engrafted *Kit*^{W-sh/W-sh} mice. **C**, MC numbers determined with toluidine blue staining in intestinal mesenteric windows from WT and CRF₂^{-/-} engrafted *Kit*^{W-sh/W-sh} mice. Data are means $\pm$ SEMs (n = 8 mice per group). Experiments were repeated in 2 independent studies. Data were analyzed by using a 2-way ANOVA: **P* < .05.

The broad inhibitory actions of CRF₂ on multiple MC degranulation stimuli and *in vivo* models led us to hypothesize that CRF₂ was targeting a common downstream mechanism in MC degranulation. Results from the present study demonstrated that MC CRF₂ expression was negatively associated with Ca²⁺ mobilization induced by a diverse array of MC stimuli, including IgE antigen, c48/80, and the muscarinic receptor 3 agonist carbachol. The ability of CRF₂ to inhibit Ca²⁺ mobilization and

degranulation provides supporting evidence that CRF₂ serves as a global regulator of MC activation. Experiments conducted under Ca²⁺-free conditions further demonstrated that CRF₂ negatively modulated cytosolic Ca²⁺ entry from intracellular stores, presumably the ER. The precise signaling pathways by which CRF₂ inhibits intracellular store Ca²⁺ release remains to be fully defined. However, results from the present study revealed an increased expression of the ER Ca²⁺-sensing protein STIM1

and the plasma membrane Ca^{2+} channels TRPC1 and Orai, which together represent the Ca^{2+} regulatory channels that play essential roles in SOCE and degranulation in MCs.^{33,36} Increased expression of STIM1, Orai, and TRPC1 has been associated with heightened Ca^{2+} mobilization and MC degranulation in food allergen-sensitized rats,³⁷ which is in line with our findings with $\text{CRF}_2^{-/-}$ BMMCs. The precise contribution of altered Ca^{2+} channel expression to CRF-mediated effects on Ca^{2+} mobilization and degranulation is unclear. Chronically increased Ca^{2+} channel activity caused by genetic loss of CRF_2 function in $\text{CRF}_2^{-/-}$ BMMCs could lead to increase protein expression of STIM1, TRPC1, and Orai1, resulting in heightened MC activity. However, that short-term pharmacologic blockade of CRF_2 and transient siRNA approaches were shown to enhance stimuli-induced MC degranulation suggests a more direct signaling event is likely involved.

Confocal microscopy revealed that CRF_2 was localized on the surfaces of MCs and intracellularly. Furthermore, intranuclear expression of CRF_2 was also observed. In comparison, CRF_1 was localized predominantly to the cell surfaces of MCs with no apparent nuclear staining patterns. Together, these findings demonstrate differential cellular localization patterns between CRF_1 and CRF_2 , but the relationship between cellular localization and divergent functions in MC activation requires further investigation. It has become increasingly clear that a number of GPCRs target to the nuclear membrane³⁸ and can regulate DNA synthesis,³⁹ transcription and gene expression,⁴⁰ and histone modification.⁴¹ To our knowledge, this represents the first evidence for differential localization of CRF receptor subtypes in MCs, and further investigations will likely provide insight into the divergent roles that CRF_1 and CRF_2 play in MC function and provide valuable insight into approaches for potential therapeutic modulation.

Precisely how the CRF receptor system is regulated to maintain MC homeostasis with regard to degranulation remains to be fully defined. We and others have shown that MCs release $\text{CRF}_1/\text{CRF}_2$ ligands CRF and urocortin under basal and stimulated conditions^{21,22} and that CRF_1 and CRF_2 receptors modulate stimuli-induced MC degranulation *in vitro* in the absence of exogenous ligands. Together, these findings demonstrate that MCs possess a functional CRF system and implicates an autocrine regulation mechanism. Such a system could explain the ability of the MC CRF system to modulate rapid cellular signaling events in the MCs, such as Ca^{2+} mobilization degranulation events. In view of the CRF system as a homeostatic regulator of the stress response, activation of CRF_1 in MCs could provide immunologic protection by enhancing MC degranulation and subsequent physiologic and immunologic responses, especially during times of acute stressful challenges. On the other hand, CRF_2 activation can provide a critical homeostatic mechanism by limiting the extent of degranulation and downstream pathophysiology. Dysfunction or imbalance of this system could lead to aberrant MC function and heightened disease states. It has been shown in cultured MCs that CRF_1 and CRF_2 expression levels can be influenced by psychological stress exogenous CRF receptor ligand, substance P, and LPS.^{18,20} There is also substantial evidence in human subjects and animal models that CRF_1 and CRF_2 expression is altered under pathologic conditions, such as urticaria,⁴² colitis,^{15,43} and chronic and early-life stress.^{44,45} The contribution of altered CRF receptor expression versus function in MC-associated disease pathogenesis remains to be fully elucidated.

In summary, the present study highlights a novel role for CRF_2 receptors expressed on MCs as negative modulators of MC degranulation in the acute response to immunologic and psychological stress. Given the critical role of MCs as immune sentinels that rapidly respond to diverse stressful stimuli, the MC CRF_2 system might represent a critical control step to limit MC degranulation and disease pathophysiology. Further understanding of the precise mechanism by which CRF_2 controls MC degranulation and associated disease pathophysiology could unveil new therapeutic targets for immune diseases linked to stress and MC hyperactivity, such as allergic inflammation and IBS.

We thank Tom Dexheimer, PhD, Manager of the Assay Development and Drug Repurposing Core (ADDRC) at Michigan State University, for his advice and technical expertise in conducting the Ca^{2+} fluorescence analyses. We also thank Darcy Honke Hulbert for the management of the mouse breeding colonies used in these experiments.

Key messages

- Loss of CRF_2 function induces exacerbated MC degranulation, IgE-mediated anaphylaxis, and psychological stress-induced intestinal barrier dysfunction.
- MC-specific CRF_2 suppresses degranulation induced by diverse MC stimuli through negative regulation of SOCE.
- Further characterization of the mechanisms by which CRF_2 negatively modulates MC activation could lead to novel therapeutic approaches for stress-related immunologic disorders associated with MC hyperactivity.

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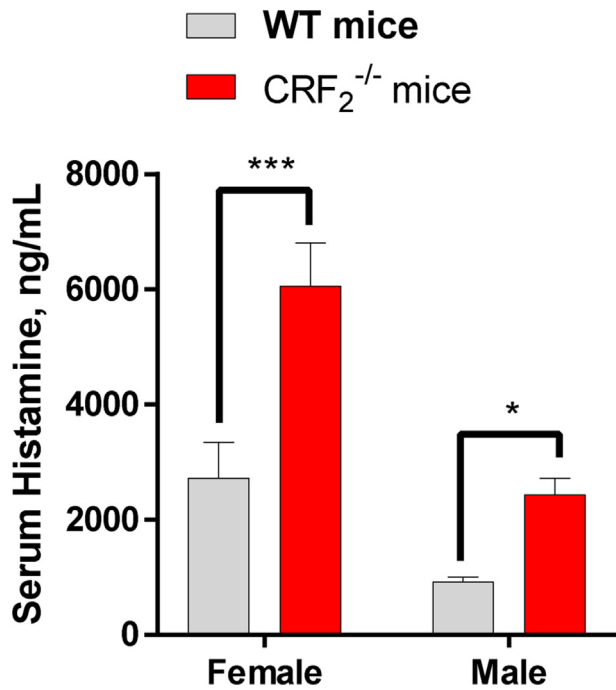


FIG E1. Serum histamine levels in female and male WT and CRF₂^{-/-} mice. Six- to 8-week-old female and male WT and CRF₂^{-/-} mice were sensitized overnight with IgE (intraperitoneal injection) and challenged with either PBS vehicle (control) or anti-IgE DNP to induce PSA. Serum histamine levels were measured at 30 minutes after vehicle or DNP challenge. Data are means $\pm$ SEMs from a representative experiment with 7 animals per group. Data were analyzed by using 2-way ANOVA: * $P < .05$ and *** $P < .001$. Experiments were repeated in 2 independent experiments.

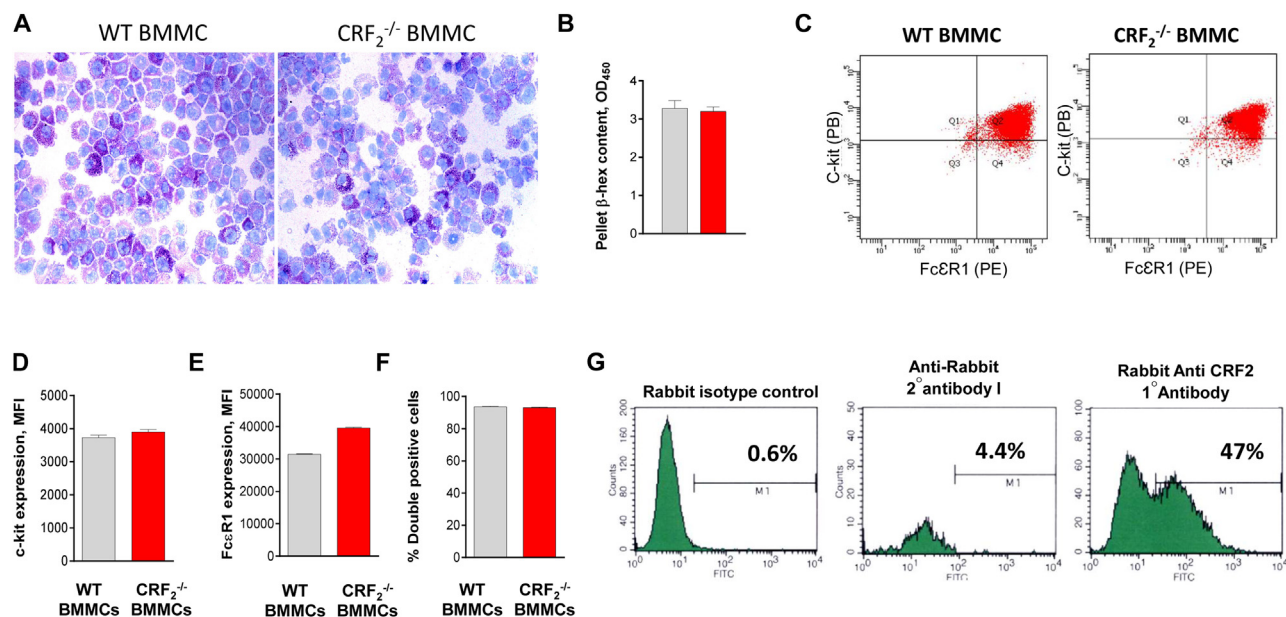


FIG E2. Phenotype and morphology of CRF₂^{-/-} BMMCs. **A**, Representative image of 6-week-old WT and CRF₂^{-/-} BMMCs stained with toluidine blue. **B**, β-Hex measured in cell pellets from WT and CRF₂^{-/-} BMMCs. **C**, Flow cytometric analysis of WT and CRF₂^{-/-} BMMCs double stained for c-kit (Pacific blue-labeled cells, *y*-axis) and FcεRI (phycoerythrin [PE]-labeled cells, *x*-axis). Mean fluorescence intensity (MFI) analysis for c-kit (**D**) and FcεRI (**E**) and percentage of c-kit/FcεRI double-positive cells (**F**). **G**, Flow cytometric analysis of BMMCs stained with primary CRF₂ antibody (*right panel*) or relevant isotype and secondary antibody controls (*left and middle panels*), as described in the Methods section.

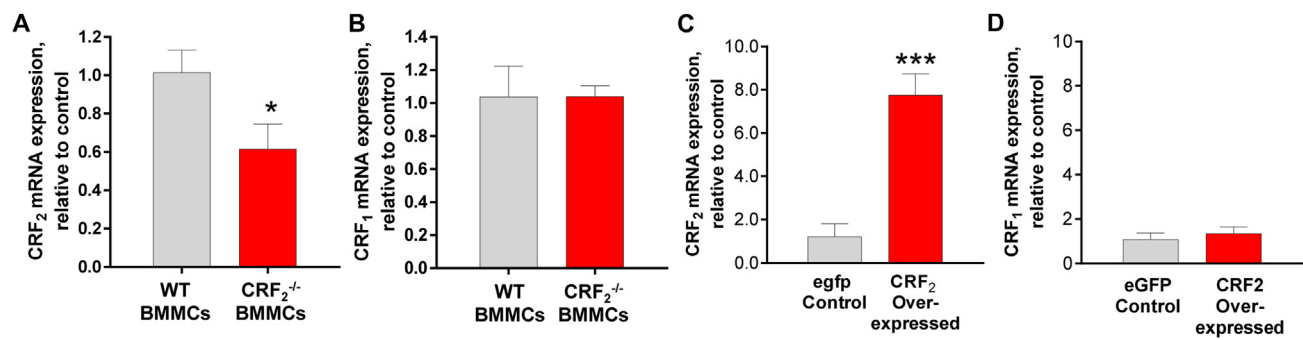


FIG E3. Expression of CRF₁ and CRF₂ in murine BMMCs and RBL-2H3 cells. CRF₂ and CRF₁ gene expression in murine WT and CRF₂^{-/-} BMMCs (A and B) and in CRF₂-overexpressed RBL-2H3 MCs (C and D). **P* < .05 and ****P* < .0001.

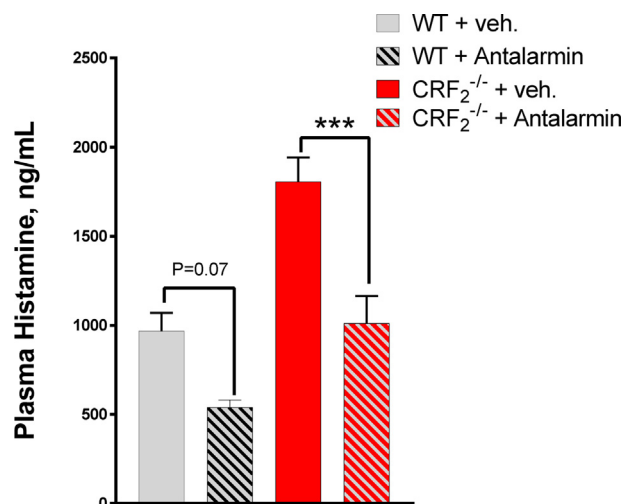


FIG E4. The CRF₁ antagonist antalarmin attenuates heightened PSA-Induced histamine levels in CRF₂^{-/-} mice. C57B6 WT male mice (6-8 weeks of age) sensitized overnight with IgE (20 μ g in 100 μ L of PBS) were administered PBS (vehicle) or the CRF₁ antagonist antalarmin (15 mg/kg administered intraperitoneally) 30 minutes before injection of DNP-HSA to induce PSA. Plasma histamine levels in mice were measured at 30 minutes after PSA. * $P < .05$ versus all other treatment groups (1-way ANOVA, $n = 5$ -6 mice per treatment).