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Letter to the Editor **β -arrestin-2 expressed in mast cells regulates ciprofloxacin-induced pseudo-allergy and IgE-mediated anaphylaxis**

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Short summary:

The adapter protein β -arrestin2 inhibits Mrgprb2 and Fc ϵ RI-mediated mast cell degranulation to attenuate pseudo-allergy and anaphylaxis. Thus, targeting β -arrestin2 in mast cells could provide novel therapeutic options for modulating pseudo-allergy and anaphylaxis.

Key words

Anaphylaxis; β -arrestin2; Ciprofloxacin; Mast cell; Pseudo-allergy

Abbreviations

β -arr2 (β -arrestin2); BMMCs (bone marrow-derived mast cells); Fc ϵ RI (high affinity IgE receptor); GPCR (G protein coupled receptor); MC (mast cell); Mrgprb2 (Mas related GPCR-b2); PCA (passive cutaneous anaphylaxis); PMCs (peritoneal mast cells); PTL (peritoneal lavage); WT (wild-type).

To the Editor:

Anaphylaxis is a life-threatening allergic reaction that results from rapid mast cell (MC) degranulation via the aggregation of high affinity IgE receptor (FcεRI). Mast cells also undergo degranulation in response to drugs approved by the US Food and Drug Administration (FDA), resulting in pseudo-allergic reactions.^{1,2} These reactions are mediated by opioids, neuromuscular blocking agents and fluoroquinolones group of antibiotics such as ciprofloxacin, which activate human MCs via Mas-related G protein coupled receptor (GPCR) X2 (MRGPRX2) and mouse MCs via Mrgprb2.^{1,2} β-arrestin2 (β-arr2) is a well-established adapter protein that contributes to GPCR desensitization³ but the possibility that it regulates MC-mediated pseudo-allergy has not been tested.⁴ Although FcεRI signaling requires tyrosine phosphorylation of Syk, the role of GPCRs on IgE-mediated MC degranulation and anaphylaxis is well documented. Transactivation of sphingosine-1-phosphate receptor 2 (SP₂) contributes to antigen/IgE-mediated MCs degranulation and anaphylaxis.^{5, 6} Furthermore, the anaphylatoxin C3a induces robust degranulation in human MCs and FcεRI-mediated cutaneous anaphylaxis is substantially reduced in C3a receptor knockout mice.^{4, 7} Based on these findings, we hypothesize that β-arr2 expressed in MCs negatively regulates both Mrgprb2-mediated pseudo-allergy and FcεRI-mediated allergy.

Using quantitative RT-PCR and Western blotting, we first confirmed the absence of β-arr2 mRNA and protein in bone marrow derived mast cells (BMMCs) generated from global β-arr2^{-/-} mice (**Fig. E1-E2**). BMMCs from both wild-type (WT) and β-arr2^{-/-} mice showed comparable levels of surface ckit and FcεRI expression and similar level of Toluidine blue and Alcian/Safranin staining (**Fig. E3-E4**). To determine the effect of β-arr2 on *in vivo* differentiation of peritoneal mast cells (PMCs), mixed cell population obtained from peritoneal lavage (PTL) were stained with toluidine blue and Alcian/Safranin and counted. As shown in

Fig. E5-E6, absence of β -arr2 had no effect on the staining properties and number of PMCs. Furthermore, transmission electron microscopy did not reveal any difference in the granule content of WT and β -arr2^{-/-} PMCs (**Fig. E7**). Toluidine blue staining of ear tissue showed no change in dermal MC number and immunofluorescence staining with Texas red Avidin (MCs) and Alexa Fluor-conjugated CD31 (Endothelial cells) showed similar compartmentalization of MCs adjacent to blood vessels in WT and β -arr2^{-/-} mice (**Fig. E8-E9**). We also generated mice with MC-specific deletion of β -arr2 by crossing *Cpa3-cre* mice with β -arr2^{fl/fl} mice. Mast cells from these mice (*Cpa-3Cre*⁺/ β -arr2^{fl/fl}) and control mice (*Cpa-3Cre*⁻/ β -arr2^{fl/fl}) (**Fig. E10**) exhibited similar FcεRI/ckit expression (**Fig. E11**), staining properties (**Fig. E12 - E13**), MC number and distribution (**Fig. E14 – E15**). These findings demonstrate that β -arr2 has no effect on the development and maturation of MCs.

Ciprofloxacin and compound 48/80 induce MC degranulation via Mrgprb2.^{1, 8} Because PMCs express Mrgprb2, we initially used mixed PTL cells as a source of PMCs for degranulation studies. Both ciprofloxacin and compound 48/80 induced β -hexosaminidase release in PTL cells but these responses were significantly enhanced in cells obtained from β -arr2^{-/-} mice (**Fig 1A**). We used a short-term culture to enrich PMCs >90%⁸ and showed that these MCs generated from β -arr2^{-/-} mice also display greater degranulation in response to ciprofloxacin and compound 48/80 when compared to cells obtained from WT mice (**Fig. 1B**). To determine the *in vivo* role of β -arr2 on pseudo-allergy, we intradermally injected ciprofloxacin or vehicle (PBS) in the ears of WT and β -arr2^{-/-} mice just after intravenous injection of Evans blue dye. Consistent with PMC degranulation, there was a significant enhancement of vascular permeability, as measured by dye extravasation, in response to ciprofloxacin in β -arr2^{-/-} mice when compared to WT mice (**Fig. 1C-D**). We also found that ciprofloxacin induced significantly

enhanced dye extravasation in $Cpa-3Cre^+/\beta\text{-arr}2^{fl/fl}$ mice when compared to control $Cpa-3Cre^-/\beta\text{-arr}2^{fl/fl}$ mice (**Fig. 1E-F**). These findings suggest that $\beta\text{-arr}2$ expressed in MCs contributes to Mrgprb2 desensitization and that its absence enhances both ciprofloxacin-induced degranulation *in vitro* and pseudo-allergy *in vivo*.

To determine the effects of $\beta\text{-arr}2$ on Fc ϵ RI signaling and mediator release, we utilized BMMCs and PMCs generated from WT and $\beta\text{-arr}2^{-/-}$ mice. The absence of $\beta\text{-arr}2$ in BMMCs had no effect on early Fc ϵ RI signaling, Syk phosphorylation or Ca^{2+} mobilization (**Fig. 2A-B**). However, antigen-induced, Fc ϵ RI-mediated degranulation, as measured by β -hexosaminidase release, was significantly enhanced in $\beta\text{-arr}2^{-/-}$ BMMCs when compared to WT-BMMCs (**Fig. 2C**). Antigen-induced β -hexosaminidase release was also significantly enhanced in $\beta\text{-arr}2^{-/-}$ PMCs when compared to WT cells (**Fig. 2D**). The fusion of lysosomal-associated membrane protein 1 (LAMP-1) with the plasma membrane upon activation is indicative of MC degranulation and can be measured by flow cytometry.⁹ As shown in **Fig. 2E** antigen triggered an increase in surface expression of LAMP-1 in WT-BMMCs but this response was significantly enhanced in $\beta\text{-arr}2^{-/-}$ cells.

We then sought to determine if enhanced antigen-induced MC degranulation in the absence of $\beta\text{-arr}2$ *in vitro* correlates with increased passive cutaneous anaphylaxis (PCA) *in vivo*. For this, ears were intradermally injected with DNP-specific IgE or vehicle, 24 h later mice were subjected to an intravenous injection of Evans blue and antigen (DNP-BSA) and dye extravasation was determined. As shown in **Fig. 2F**, antigen caused ~2-fold increase in dye extravasation when compared to PBS control in WT mice. However, this response was significantly enhanced in $\beta\text{-arr}2^{-/-}$ mice. Antigen-induced PCA was also enhanced in $Cpa-3Cre^+/\beta\text{-arr}2^{fl/fl}$ mice when compared to control $Cpa-3Cre^-/\beta\text{-arr}2^{fl/fl}$ mice (**Fig. 2G**). These

findings demonstrate that β -arr2 inhibits antigen-induced MC degranulation *in vitro* and PCA *in vivo* via modification of signaling components downstream of Syk phosphorylation and Ca^{2+} mobilization. To determine if the effects of β -arr2 on MC degranulation is specific for Mrgprb2 and Fc ϵ RI, BMMCs and PMCs from WT and β -arr2^{-/-} mice were stimulated with PMA/ Ca^{2+} ionophore and β -hexosaminidase release was determined. Surprisingly, β -arr2^{-/-} MCs exhibited enhanced degranulation when compared to WT cells (**Fig. E16 - E17**). These findings suggest that β -arr2 regulates Mrgprb2 and Fc ϵ RI-mediated MC degranulation via the modification of a shared target downstream of Ca^{2+} mobilization.

The novel finding of the present study is that the same adapter protein, β -arr2, negatively regulates both Mrgprb2 and Fc ϵ RI-mediated MC degranulation to attenuate drug-induced pseudo-allergy and IgE-mediated anaphylaxis.^{1, 2, 4-7} We also demonstrated that absence of β -arr2 results in enhanced degranulation in response to Ca^{2+} ionophore. This suggests that β -arr2 plays a global role in regulating MC responsiveness to any stimuli that induce degranulation. Thus, detailed understanding of the molecular mechanism via which β -arr2 regulates MC function may provide a novel strategy for the modulation of diseases that are mediated by MCs.

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Figure Legends:

Figure 1: β -arrestin2 regulates ciprofloxacin-induced MC degranulation *in vitro* and pseudo-allergy *in vivo*. Degranulation in WT and β -arr2^{-/-} (A) PTLs and (B) PMCs upon stimulation with ciprofloxacin (200 μ g/mL) or compound 48/80 (10 μ g/mL). (C) WT and β -arr2^{-/-} mice were intradermally injected with ciprofloxacin (400 μ g/mL) or PBS and Evan's blue dye extravasation was determined. N=5-6. (D) A representative image is shown. (E) Evan blue extravasation in *Cpa3-Cre*⁻/ *β -arr2*^{fl/fl} and *Cpa3-Cre*⁺/ *β -arr2*^{fl/fl} mice. N=5-6 (F) A representative image is shown. Error bars are SD. ***, P <0.001; **, P <0.01 and *, P <0.05 (non-parametric t-Test).

Figure 2: β -arrestin2 regulates IgE-mediated MC degranulation *in vitro* and cutaneous anaphylaxis *in vivo*. IgE primed WT and β -arr2^{-/-} BMMCs were stimulated with antigen (DNP-BSA) and (A) Syk phosphorylation (B) Ca²⁺ mobilization were determined. Antigen-induced β -hexosaminidase release in (C) WT and β -arr2^{-/-} BMMCs and (D) PMCs. Antigen-induced LAMP-1 expression in WT and β -arr2^{-/-} BMMCs (E). Mice were sensitized with intradermal injection of IgE (20 ng) and Evan's blue extravasation was determined following intravenous injecting of DNP-BSA in (F) WT and β -arr2^{-/-} (G) *Cpa3-Cre*⁻/ *β -arr2*^{fl/fl} and *Cpa3-Cre*⁺/ *β -arr2*^{fl/fl} mice. N= 5-6. Error bars are SD ***, P <0.001; **, P <0.01 and *, P <0.05 (non-parametric t-Test).

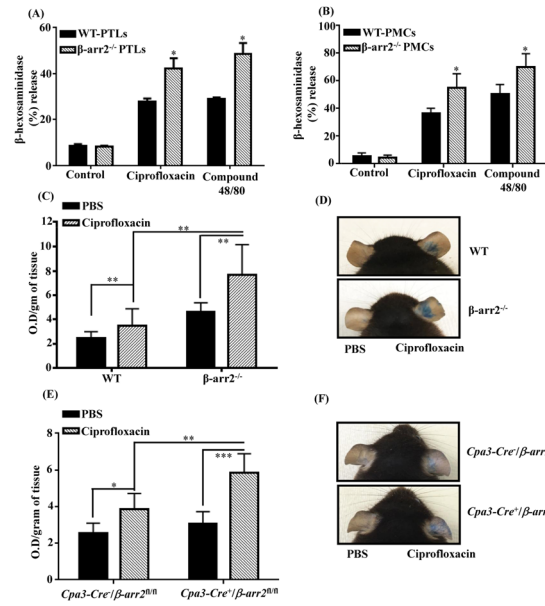


Figure 1

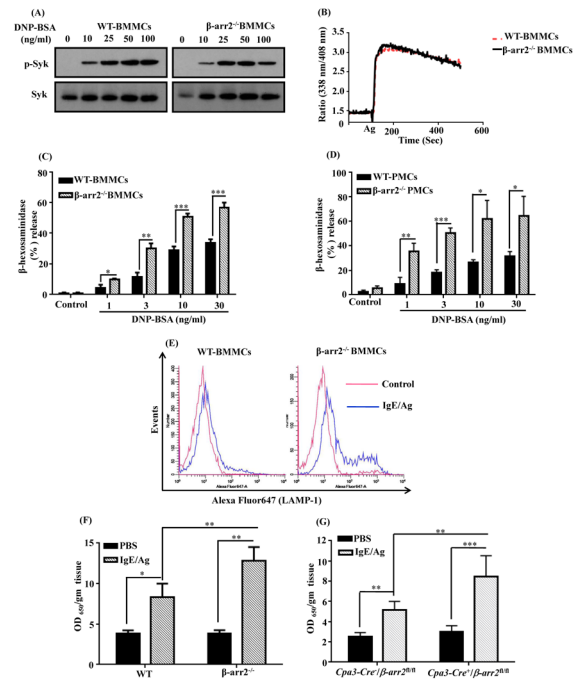


Figure 2

SUPPLEMENTAL MATERIALS

Reagents

All cell culture reagents and DNP-specific mouse IgE (SPE-7) were purchased from Invitrogen (Carlsbad, CA). Recombinant mouse interleukin-3 (IL-3) and stem cell factor (SCF) were purchased from Peprotech (Rocky Hill, NJ). DNP-BSA, p-nitrophenyl-N-acetyl- β -D-glucosamine (PNAG), Ionomycin, Phorbol 12-myristate 13-acetate (PMA) and Evan's blue dye were obtained from Sigma-Aldrich (St. Louis, MO). Phycoerythrin (PE) conjugated c-kit and Fluorescein isothiocyanate (FITC) conjugated Fc ϵ RI antibodies were acquired from eBiosciences (San Diego, CA). Alexa Fluor 647-conjugated anti mouse LAMP-1 and Alexa Fluor 488-conjugated anti-mouse CD31 antibodies were purchased from BioLegend (San Diego, CA). Antibodies against β -arrestin2, β -actin, p-Syk, Syk, and HRP-labeled goat anti-rabbit IgG were obtained from Cell Signaling Technology (Danvers, MA). SuperSignal® West Pico Chemiluminiscent Substrate was from Thermo Scientific (Rockford, IL). Indo-1 AM and Texas Red-conjugated Avidin was purchased from Molecular Probes (Eugene, OR). Compound 48/80 was obtained from AnaSpec (Fremont, CA) and ciprofloxacin was from Acros Organics, Fisher Scientific (Hampton, NH).

Mice

C57BL/6 (WT) and β -arrestin2 global knockout (β -arr2^{-/-}) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). β -arrestin2^{flox/flox} (β -arr2^{fl/fl}) mice were a kind gift from Drs. Robert Lefkowitz and Julia Walker (Duke University Medical Center, Durham, NC), and *Cpa3-Cre* mice were generously provided by Dr. Stephen Galli (Stanford University, Stanford, CA). Mice were housed in pathogen-free cages on autoclaved hardwood bedding. Mice with

mast cell-specific deletion of β -arr2 ($Cpa3-Cre^+/\beta$ -arr2^{fl/fl}) were generated by crossing $Cpa3-Cre^+$ mice with β -arr2^{fl/fl} mice. $Cpa3-Cre^+/\beta$ -arr2^{fl/fl} mice were screened and selected based on Cre and flox specific PCR. $Cpa3-Cre^-/\beta$ -arr2^{fl/fl} mice were used as respective controls. Mice (7-12) weeks old were used in the entire study. All experiments were approved by Institutional Animal Care and Use Committee at The University of Pennsylvania.

Cells

Peritoneal MCs (PMCs) were purified as described previously but with some modifications.^{E1} The peritoneal cavity was lavaged with Hank's Balanced Salt Solution (HBSS) (10 mL) supplemented with 3% fetal calf serum (FCS). Cells (0.5×10^6 /mL) were cultured in Iscove's Modified Medium (IMDM) containing 10 ng/mL recombinant mIL-3 and 30 ng/mL mSCF for 48 h. Non-adherent cells were removed and adherent cells were cultured in fresh medium for an additional 10-14 days. Floating cells were used for experiments as PMCs. Bone marrow-derived mast cells (BMMCs) were obtained by *in vitro* differentiation of bone marrow cells obtained from WT, β -arr2^{-/-}, $Cpa3-Cre^-/\beta$ -arr2^{fl/fl} and $Cpa3-Cre^+/\beta$ -arr2^{fl/fl} femurs and cultured as described previously.^{E2} The purity of BMMCs was usually > 90% as determined by flow cytometry (BD LSR II flow cytometer, BD Biosciences) using anti-c-kit-PE and anti-FcεRI-FITC antibodies.

Mast cell staining

Toluidine blue and Alcian/Safranin staining were performed on the cultured BMMCs (5×10^4) and freshly isolated peritoneal lavage cells (1×10^5) as described previously^{E3}. Images were captured using a Nikon E600 microscope with a digital camera attached at 40X magnification and analyzed using Nikon NIS Elements software. Mast cell numbers were counted manually.³ Ears from the WT, β -arr2^{-/-}, $Cpa3-Cre^{-/-}/\beta$ -arr2^{fl/fl} and $Cpa3-Cre^{+}/\beta$ -arr2^{fl/fl} mice were dissected out and dermal sheets were excised followed by fixation in formaldehyde overnight. Dermal sheets were stained with Texas Red-Avidin and Alexa Fluor 488-conjugated CD31 as described previously^{E4}. Images were captured using a Nikon A1R laser scanning confocal microscope with a 40X water objective (NA=1.2) and analyzed using Nikon NIS Elements software.

Transmission Electron Microscopy

Transmission electron microscopy was performed at the Electron Microscopy Resource Laboratory, Perelman School of Medicine, University of Pennsylvania. Freshly isolated peritoneal lavage cells from WT and β -arr2^{-/-} mice were washed and fixed (2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.4) overnight at 4°C. After subsequent buffer washes, the samples were post-fixed in 2.0 % osmium tetroxide for 1 h at room temperature, and rinsed in distilled H₂O prior to *en bloc* staining with 2% uranyl acetate. After dehydration through a graded ethanol series, the tissue was infiltrated and embedded in EMbed-812 (Electron Microscopy Sciences, Fort Washington, PA). Thin sections were stained with uranyl acetate and lead citrate, and examined with a JEOL 1010 electron microscope fitted with a Hamamatsu digital camera and AMT Advantage image capture software.

FcεRI and ckit expression

For analysis of cell surface receptor expression, WT, β -arr2^{-/-}, *Cpa3-Cre*⁻/ β -arr2^{fl/fl} and *Cpa3-Cre*⁺/ β -arr2^{fl/fl} BMMCs (0.5×10^6) were washed with FACS buffer (PBS containing 2% FCS and 0.02% sodium azide) and stained with anti-c-kit-PE and anti-FcεRI-FITC for 1h at 4°C. Cells were washed and fixed in 1.5% paraformaldehyde solution. The samples were acquired in a BD LSR II flow cytometer (BD Biosciences) and analyzed by WinList software.^{E2}

Real-time PCR

Total mRNA was isolated from WT, β -arr2^{-/-}, *Cpa3-Cre*⁻/ β -arr2^{fl/fl} and *Cpa3-Cre*⁺/ β -arr2^{fl/fl} BMMCs using the RNeasy Mini Kit as per the manufacturer's instructions. Real time PCR analysis was performed by mixing cDNA with 2× TaqMan master mix and running samples on an Applied Biosystems 7500 Fast Real Time PCR System. Relative quantification of β -arr2 gene was normalized to GAPDH and expressed as a fold change using the comparative cycle threshold (CT) method.^{E5}

Western Blotting

IgE-primed BMMCs were stimulated with different concentrations of antigen (DNP-BSA) for 5 min and lysed. Protein was separated in SDS-PAGE (10 %), immunoblotted onto PVDF membranes, blocked and probed with anti-p-Syk antibody (1:1000). Following incubation by HRP-conjugated secondary antibody, membrane was developed using the SuperSignal® West Pico Chemiluminiscent Substrate. The membrane were stripped and reprobed with total Syk

antibody. Expression of β -arrestin-2 and β -actin in WT and β -arr^{2-/-} BMMCs were performed similarly.

Calcium mobilization

BMMCs (1.5×10^6) were preincubated with anti-DNP mouse IgE antibody (1 μ g/mL, 18h). Cells were washed in HEPES-buffered saline containing 1% BSA, loaded with 1 μ M indo-1 AM for 30 min, washed, and resuspended in 1.5 mL of HEPES-buffered saline. Ca^{2+} mobilization was measured by stimulating with 100 ng/mL DNP-BSA in a Hitachi F-2500 spectrophotometer with an excitation wavelength of 338 nm and an emission wavelength of 408 nm.

Degranulation

BMMCs (5×10^4 /well) and PMCs (1×10^4 /well) were sensitized with anti-DNP mouse IgE antibody (1 μ g/mL, 18h) and incubated with different concentrations of antigen (DNP-BSA, 0-30 ng/mL) at 37°C for 30 min. For total β -hexosaminidase release, unstimulated cells were lysed in 50 μ L of 0.1% Triton X-100. Aliquots (20 μ L) of supernatant or cell lysates were incubated with 20 μ L of 1 mM p-nitrophenyl-N-acetyl- β -D-glucosamine for 1 h at 37°C. The reaction was stopped by adding 250 μ L of a 0.1 M Na_2CO_3 /0.1 M NaHCO_3 buffer and absorbance was measured at 405 nm.^{E2} For PMA/ionomycin stimulation, BMMCs and PMCs were exposed to PMA (50 nM for 5 min) and subsequently stimulated with ionomycin (0.5 - 1.5 μ g/mL) for additional 30 min and β -hexosaminidase release was determined. Degranulation in peritoneal lavage cells (1.5×10^5 /well) and PMCs from WT and β -arr^{2-/-} mice was performed similarly after stimulation with ciprofloxacin (200 μ g/mL) or compound 48/80 (10 μ g/mL). Flow

cytometry was also used to determine degranulation in BMMCs. For this, IgE-primed cells (0.5×10^6) were stimulated with DNP-BSA (10 ng/mL) for 10 min, washed and exposed to anti-LAMP-1 Alexa Fluor 647 antibody for 1 h at 4°C. Cell surface expression of LAMP-1 was determined by flow cytometry.^{E6}

Pseudo-allergy and Passive cutaneous anaphylaxis (PCA)

For pseudo-allergic drug reaction, WT, β -arr2^{-/-}, $Cpa3-Cre^+/\beta$ -arr2^{fl/fl} or $Cpa3-Cre^-/\beta$ -arr2^{fl/fl} mice were intravenously injected with 1% Evans blue followed by intradermal injection of 30 μ L ciprofloxacin (400 μ g/mL) in the right ear and PBS in the left ear (Control). After 30 min, mice were euthanized; the ears were removed, weighed, dissolved in 500 μ L formamide and incubated at 55°C overnight. Supernatant was collected and absorbance was measured at 650 nm. For PCA, mice were intradermally injected with IgE (20 ng) into the right ear or PBS in the left ear (control). After 24 h, mice were challenged with an intravenous injection of 100 μ g DNP-BSA in 200 μ L PBS containing 1% Evans blue, and dye extravasation was determined as described for ciprofloxacin.

Statistical Analysis

All *in vitro* data are mean $\pm$ standard deviation (SD) value derived from three independent experiments. Statistical significance was determined by non-parametric t-test. Significant differences were set at * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ and analyzed by GraphPad Prism version 6.07.

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Supporting Figure Legends

Figure E1: Real-time PCR analysis was performed to determine the mRNA expression of β -arr2 in WT and β -arr2^{-/-} BMMCs. GAPDH was used for normalization. Data is represented as mean $\pm$ SD. *** $P \leq 0.001$ (non-parametric t-Test).

Figure E2: Western blotting was performed to determine expression of β -arr2 in WT and β -arr2^{-/-} BMMCs using anti- β arr2 antibody. β -actin antibody was used for normalization.

Figure E3: Surface expression of Fc ϵ RI and c-kit on WT and β -arr2^{-/-} BMMCs was determined by flow cytometry. Representative traces are shown.

Figure E4: WT and β -arr2^{-/-} BMMCs (50,000) were cytopun, dried and stained with Toluidine blue and Alcian/Safranin. The images were acquired at 40X resolution.

Figure E5: Freshly isolated peritoneal lavage cells (100,000) from WT and β -arr2^{-/-} mice were cytopun, dried and stained with Toluidine blue and Alcian/Safranin. The images were acquired at 40X resolution.

Figure E6: Toluidine blue and Alcian/Safranin stained peritoneal lavage cells were counted manually in light microscope and presented as mean $\pm$ SD of 10 fields randomly selected from three different mice. NS, not significant (non-parametric t-Test).

Figure E7: Freshly isolated peritoneal lavage cells from WT and β -arr2^{-/-} mice were used for transmission electron microscopy to visualize MC granules. 'N' is depicted as mast cell nucleus and 'G' is depicted as mast cell granules.

Figure E8: Paraffin-embedded ear sections from WT and β -arr2^{-/-} mice were stained with Toluidine blue. MC number in the individual field was counted manually in a light microscope and presented as mean $\pm$ SD of three different mice. NS, not significant (non-parametric t-Test).

Figure E9: Representative confocal images of whole ear mounts of WT and β -arr2^{-/-} mice stained with Texas Red Avidin (Red, mast cells) and Alexa Fluor 488 conjugated CD31 (Green, Endothelial cells). Bars = 50 μ m

Figure E10: Real-time PCR analysis of mRNA expression of β -arr2 in *Cpa3-Cre*⁻/ β -arr2^{fl/fl} and *Cpa3-Cre*⁺/ β -arr2^{fl/fl} BMMCs. Data is represented as mean $\pm$ SD. *** $P \leq 0.001$ (non-parametric t-Test).

Figure E11: Expression of FcεRI and c-kit on *Cpa3-Cre⁻/β-arr2^{fl/fl}* and *Cpa3-Cre⁺/β-arr2^{fl/fl}* BMMCs was determined by flow cytometry. Representative traces are shown.

Figure E12: *Cpa3-Cre⁻/β-arr2^{fl/fl}* and *Cpa3-Cre⁺/β-arr2^{fl/fl}* BMMCs were stained with Toluidine blue and Alcian/Safranin. The images were acquired at 40X resolution.

Figure E13: Freshly isolated peritoneal lavage cells from *Cpa3-Cre⁻/β-arr2^{fl/fl}* and *Cpa3-Cre⁺/β-arr2^{fl/fl}* mice were stained with Toluidine blue and Alcian/Safranin. The images were acquired at 40X resolution.

Figure E14: Paraffin-embedded ear sections from *Cpa3-Cre⁻/β-arr2^{fl/fl}* and *Cpa3-Cre⁺/β-arr2^{fl/fl}* mice were stained with Toluidine blue and MC number was counted and presented as mean ± SD of three different mice. NS, not significant (non-parametric t-Test).

Figure E15: Representative confocal images of whole ear mounts from *Cpa3-Cre⁻/β-arr2^{fl/fl}* and *Cpa3-Cre⁺/β-arr2^{fl/fl}* mice stained with Texas Red Avidin (MC) and Alexa Fluor 488 conjugated CD31 (Endothelial cells)

Figure E16: WT and β -arr2^{-/-} BMMCs (5×10^4) were preincubated with PMA (50 nM, 5 min), stimulated with ionomycin (0 – 1.5 μ g/mL) and β -hexosaminidase release was determined. Data are expressed as mean $\pm$ SD. *** P < 0.001 and ** P < 0.01 (non-parametric t-test).

Figure E17: WT and β -arr2^{-/-} PMCs (1×10^4) were stimulated with PMA/ionomycin (0 – 1.5 μ g/mL) and β -hexosaminidase release was determined. Data are expressed as mean $\pm$ SD. *** P < 0.001 and ** P < 0.01 (non-parametric t-test).

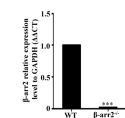


Figure E1

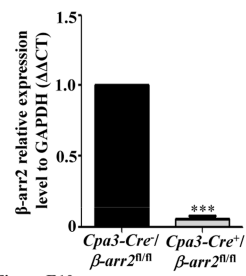


Figure E10

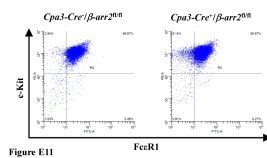


Figure E11

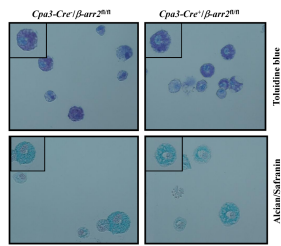


Figure E12

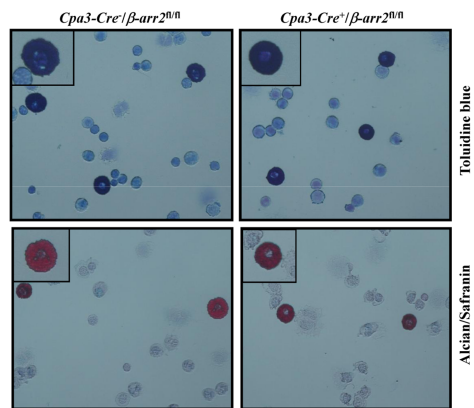


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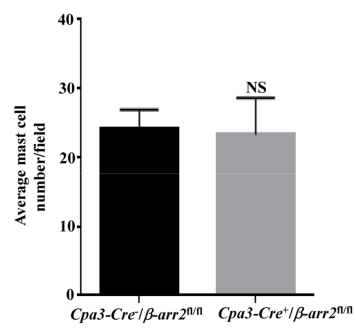


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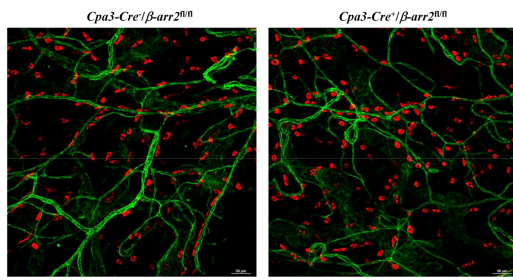


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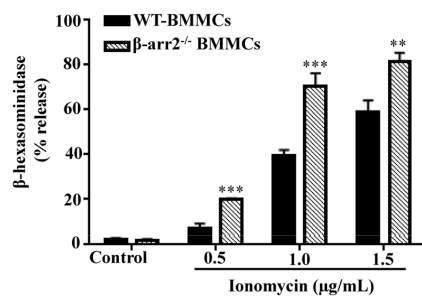


Figure E16

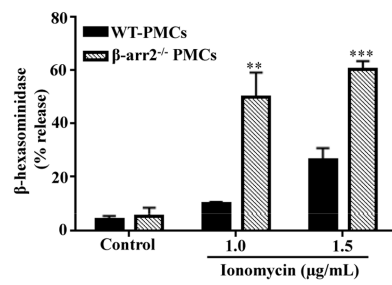


Figure E17

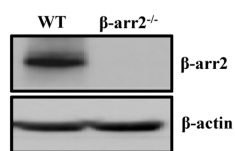


Figure E2

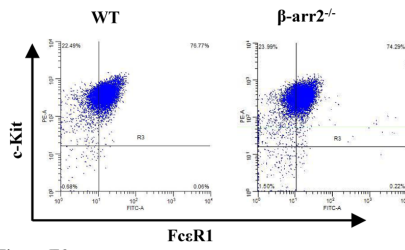


Figure E3

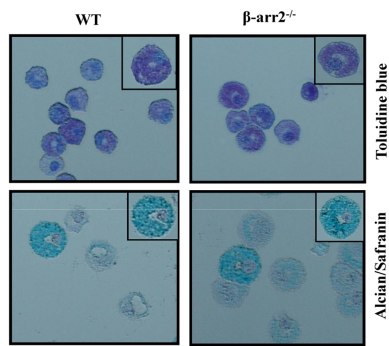


Figure E4

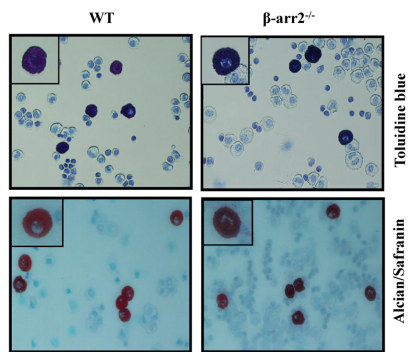


Figure E5

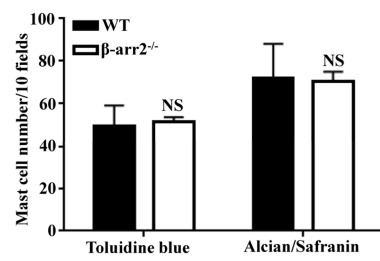


Figure E6

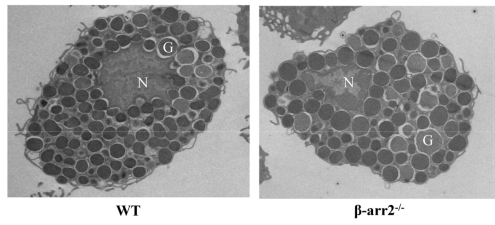


Figure E7

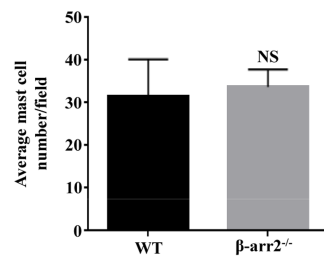


Figure E8

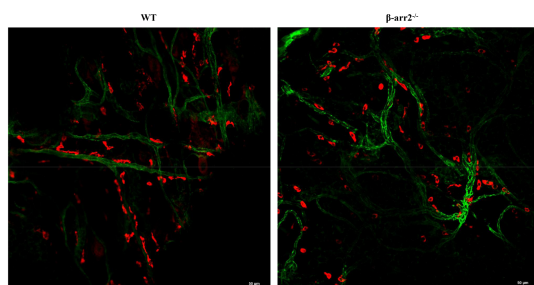


Figure E9