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Platelet-Activating Factor–Induced Reduction in Contact Hypersensitivity Responses Is Mediated by Mast Cells via Cyclooxygenase-2–Dependent Mechanisms

Jesus A. Ocana,* Eric Romer,[†] Ravi Sahu,[†] Sven-Christian Pawelzik,[‡] Garret A. FitzGerald,[‡] Mark H. Kaplan,[§] and Jeffrey B. Travers^{†,¶,||}

Platelet-activating factor (PAF) stimulates numerous cell types via activation of the G protein–coupled PAF receptor (PAFR). PAFR activation not only induces acute proinflammatory responses, but it also induces delayed systemic immunosuppressive effects by modulating host immunity. Although enzymatic synthesis and degradation of PAF are tightly regulated, oxidative stressors, such as UVB, chemotherapy, and cigarette smoke, can generate PAF and PAF-like molecules in an unregulated fashion via the oxidation of membrane phospholipids. Recent studies have demonstrated the relevance of the mast cell (MC) PAFR in PAFR-induced systemic immunosuppression. The current study was designed to determine the exact mechanisms and mediators involved in MC PAFR-mediated systemic immunosuppression. By using a contact hypersensitivity model, the MC PAFR was not only found to be necessary, but also sufficient to mediate the immunosuppressive effects of systemic PAF. Furthermore, activation of the MC PAFR induces MC-derived histamine and PGE₂ release. Importantly, PAFR-mediated systemic immunosuppression was defective in mice that lacked MCs, or in MC-deficient mice transplanted with histidine decarboxylase– or cyclooxygenase-2–deficient MCs. Lastly, it was found that PGs could modulate MC migration to draining lymph nodes. These results support the hypothesis that MC PAFR activation promotes the immunosuppressive effects of PAF in part through histamine- and PGE₂-dependent mechanisms. *The Journal of Immunology*, 2018, 200: 4004–4011.

Platelet-activating factor (PAF; 1-hexadecyl-2-acetyl-glycerophosphocholine) is a soluble lipid mediator of both local acute inflammation as well as delayed systemic immunosuppression (1–7). PAF binds a single G protein–coupled receptor, PAF receptor (PAFR; *Ptafr*), expressed in many cell types, including platelets, neutrophils, macrophages, dendritic cells, and mast cells (MCs) (8–11). PAF stimulates the activation of platelets (12–14), induces macrophage, neutrophil, and eosinophil chemoattraction (15–17) and neutrophil extracellular trap release (18), and it has recently been discovered to stimulate MC

migration to lymph nodes (LNs) (19). Whereas the proinflammatory effects of PAF are well characterized, the immunosuppressive effects of PAF remain an active area of study. It has been found that exogenous PAF is sufficient to induce systemic immunosuppression and that the immunosuppressive effects of UVB are mediated by PAF (13). Our laboratory characterized the latter effect in demonstrating that UVB generates PAF-like phospholipids (20) that induce systemic immunosuppression. Similar to UVB, other oxidative stressors have demonstrated PAFR-dependent systemic immunosuppression, including cigarette smoke, jet fuel, and photodynamic therapy (10, 11).

MCs are long-lived hematopoietic cells that initiate allergic responses upon recognition of pathogenic stimuli. MCs store prepackaged vesicles containing inflammatory mediators, such as histamine, serotonin, and proteases, which are released following MC activation. MCs can also synthesize and secrete cytokines and chemokines, as well as eicosanoids (21–23). In addition to mediator release, MC migration to draining LNs has been found to induce immune responses (24). Thus, MCs play an obligate proinflammatory role in many immune responses.

In contrast to these proinflammatory functions, MCs have also been shown to attenuate immune responses (25–27). MC IL-10 release has been shown to limit pathology from contact dermatitis and chronic UVB irradiation (28). MCs have also been shown to contribute to transplant tolerance (29). Additionally, experiments have demonstrated that MCs induce regulatory T cell (Treg) differentiation via TGF- β –dependent mechanisms when cocultured with naive T cells (30), and they augment the suppressor activity of myeloid-derived suppressor cells (31, 32). Moreover, MCs fine-tune immune responses by transiently attenuating Treg function through histamine receptor H1 activation (33) and inducing Treg recruitment by H4 receptor activation (34). Furthermore, MCs attenuate antitumor immunity to promote skin tumorigenesis (35, 36), and they mobilize Tregs and myeloid-derived suppressor cells in the tumor microenvironment (37).

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Abbreviations used in this article: BMDC, bone marrow–derived MC; CHS, contact hypersensitivity; COX-2, cyclooxygenase-2; CPAF, 1-hexadecyl-2-*N*-methylcarbamoyl glycerophosphocholine; DNFB, dinitrofluorobenzene; EIA, enzyme immunoassay; HDC, histidine decarboxylase; KO, knockout; LN, lymph node; MC, mast cell; PAF, platelet-activating factor; PAFR, PAF receptor; qRT-PCR, quantitative RT-PCR; RT, room temperature; Treg, regulatory T cell; WT, wild-type.

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MCs mediate the immunosuppressive effects of UV light, and s.c. injected histamine, the primary mediator released by MCs, had a similar effect (38). Additionally, PAFR expression on MCs was necessary for UVB-induced immunosuppression in a process mediated in part by migration of MCs to draining LNs via a CXCR4 signaling axis (19). However, the contribution of other non-MC PAFRs is unknown. Lastly, previous studies have shown that cyclooxygenase-2 (COX-2) inhibitors can attenuate the immunosuppressive effects of PAF (39, 40), and that the E prostanoide 4 receptor is necessary for the immunosuppressive effects of UV light (41). As MCs have been demonstrated to generate a number of eicosanoids (21–23), the role of MC-derived PGs may be relevant to the immunosuppressive mechanisms of PAF. The present study set out to further delineate the link between PAF, MCs, histamine, and COX-2-generated PG synthesis during UVB-induced systemic immunosuppression.

Materials and Methods

Reagents and cells

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise. COX-2 inhibitor (SC-236), TGF- β inhibitor (LY364947), and a histamine enzyme immunoassay (EIA) kit were obtained from Cayman Chemical (Ann Arbor, MI). PGE₂ EIA was obtained from R&D Systems (Minneapolis, MN). Quantitative PCR reagents were obtained from Invitrogen (Carlsbad, CA). Primers for quantitative PCR were obtained from Integrated DNA Technologies (Coralville, IA). Abs for immunoblot analysis were obtained from Abcam (Cambridge, MA; histidine decarboxylase [HDC], rabbit polyclonal no. ab37291; COX-2, rabbit monoclonal no. ab62331). MCs were obtained by culturing murine bone marrow in 10% FBS and IL-3 (10 ng/ml; PeproTech, Rocky Hill, NJ) containing IMDM media for 4–8 wk. Cell cultures contained MC populations (Fc ϵ R⁺, c-Kit⁺) >90% as measured by flow cytometry, using Abs from eBioscience (San Diego, CA) and BD Biosciences (San Jose, CA). Cells used for in vitro studies were incubated in 1 ml of 10% FBS containing IMDM media supplemented with 10 ng/ml IL-3 in 12-well plates.

Mice

C57BL/6 (wild-type [WT]; Charles River Laboratories, Wilmington, MA) and C57BL/6 *Kit^{Wsh/Wsh}* (*Wsh*) (B6.Cg-*Kit^{W-sh}*/HNIhrJaeBsmGlij; The Jackson Laboratory, Bar Harbor, ME) mice were obtained commercially. *Ptafr^{-/-}* (PAFR knockout [KO]) (from Prof. T. Shimizu, University of Tokyo, Tokyo, Japan) and *Hdc^{-/-}* (HDC KO) (from Dr. H. Ohtsu, Tohoku University, Miyagi, Japan) mice on a C57BL/6 background were kept under pathogen-free conditions. Bone marrow from *Mcpt5-cre/Ptgs2^{flax/flax}* and *Ptgs2^{flax/flax}* mice for MC transplantation were obtained from mice previously described (42, 43). BoyJ mice were provided by the In Vivo Therapeutics Core (Indiana University School of Medicine, Indianapolis, IN). Both male and female 6- to 14-wk-old mice were used for the experiments. All mice were housed under specific pathogen-free conditions at the Indiana University School of Medicine or, in the case of *Mcpt5-cre/Ptgs2^{flax/flax}* and *Ptgs2^{flax/flax}* mice, the University of Pennsylvania. All procedures were approved by the Animal Care and Use Committees of respective institutions.

MC transplantation and contact hypersensitivity assay

MCs (10⁶) were injected s.c. into two rows of four injection sites in a 1- by 2-cm area on the shaved dorsal skin of 6- to 8-wk-old *Wsh* or PAFR KO *Wsh* mice. At 6 wk after implantation, mice were used for experiments. For the contact hypersensitivity (CHS) assay, mice were either treated on shaved dorsal skin with vehicle, UVB (7.5 kJ/m² using a Philips F20T12/UV-B lamp), histamine (200 μ g s.c.), or 1-hexadecyl-2-N-methylcarbamoyl glycerophosphocholine (CPAF; 200 ng i.p.). Five days after treatment, 25 μ l of 0.5% dinitrofluorobenzene (DNFB; in 4:1 acetone/olive oil) was applied to shaved dorsal skin at least 2 cm away from the UVB-treated area. Nine days later ear thickness was measured using a constant pressure analog thickness gauge (Peacock model G, 0.4 N, mm $\times$ 10⁻³). Subsequently, one ear was treated with 10 μ l of 0.5% DNFB whereas the other ear was treated with vehicle. After 24 h, ear thickness was measured again in these mice. The difference in ear swelling between DNFB- and vehicle-treated ears was normalized to the ear swelling in vehicle-treated mice (see Fig. 1A for details).

Histology

Dorsal skin samples or LNs from mice were formalin fixed for 24 h before storage in ethanol. Specimens were paraffin embedded, sectioned, and

stained for MCs using acidified toluidine blue by the Indiana University School of Medicine Histology Core (44). MC numbers were quantified by counting 10 high-power fields ($\times$ 600).

Quantitative RT-PCR

Total RNA was extracted from treated MCs using the RNeasy kit (Qiagen). In brief, tissue was homogenized in RLT buffer containing 2-ME by pipetting and QIAshredder (Qiagen). Purified RNA was quantitated with the NanoDrop 2000 (Thermo Fisher Scientific, Lafayette, CO). Reverse transcription of whole RNA was done using SuperScript cDNA synthesis kit (Invitrogen) with random hexamers. Quantitative RT-PCR (qRT-PCR) was performed for *Ptgs2* and *Hdc* against *Gapdh* as the endogenous control using the $\Delta\Delta$ Ct method on a StepOne real-time PCR machine (Applied Biosystems, Foster City, CA). Each assay was performed in triplicate in a 10- μ l reaction volume with TaqMan master mix (SABiosciences, Frederick, MD), 1 ng of cDNA, primers at 500 nM, and probe at 250 nM.

Flow cytometry

Cells (10⁶) were taken from culture and plated in a 96-well plate in FACS buffer (PBS, 1% BSA, 0.1% NaN₃) for staining. Cells were incubated with Fc Block (no. 553142; BD Biosciences) for 15 min and then stained with 1:100 or 1:200 dilutions of conjugated primary Ab (CD45.1-PE, c-Kit-allophycocyanin, CD4-PerCP, Fc ϵ R-PE) for 30 min. Cells were then washed and resuspended in PBS to be stained with viability dye (eFluor 780) at a dilution of 1:1000. Cells were then washed and resuspended in FACS buffer to a concentration of $\sim$ 10⁵ cells/100 μ l for analysis on the Invitrogen Attune cytometer or the BD LSR II.

Western blotting and densitometry

Cells (6 $\times$ 10⁶) were harvested and washed twice with PBS. Cell pellets were then lysed with 150 μ l of lysis buffer (1% Triton X-100, 10 mM Tris base, 150 mM NaCl, 1% protease inhibitor mixture [pH 7.4]; Sigma-Aldrich) and centrifuged for 15 min at 400 $\times$ g at 4°C. Supernatants were collected (120 μ l), and the protein concentration was measured by Bio-Rad spectrophotometry methods. Protein (30–100 μ g) with 2-ME-containing reducing SDS sample buffer was then loaded into a 10% acrylamide gel and run for 45 min under a constant 400 mA. Proteins were then transferred onto a nitrocellulose membrane for 1 h at a constant 100 V at 4°C. The membrane was then cut and incubated with blocking buffer (5% powdered milk, 0.1% Tween 20, in PBS) for 2 h at room temperature (RT) or overnight at 4°C. Membranes were then incubated with the appropriate amount of Ab for 2 h at RT or overnight at 4°C. Membranes were then washed with TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween 20) four times for 5 min each before incubation with HRP-conjugated secondary Ab (1:5000) for 30 min at RT. Membranes were then washed with TBST four times for 5 min per wash before incubating the membranes with HRP substrate (Millipore, Billerica, MA) for 1 min. Films were then exposed at serial time points and developed. Radiographs were then scanned, digitized, and quantitated by pixel densitometry using ImageJ. Expression by densitometry was calculated by pixel density of the gene of interest divided by GAPDH pixel density and normalized to vehicle ratio.

ELISA

Cells (3 $\times$ 10⁶) were plated and treated for the described length of time. Supernatants were then collected from suspensions centrifuged at 400 $\times$ g for 10 min. These supernatants were then diluted according to the detectable range of the ELISA (1:10 or 1:100) in media or analysis buffer.

Statistical analysis

All statistical calculations were performed using Prism 6. Statistical comparisons are described in the figure legend for each experiment. The data represent mean values with SEM. Differences were considered statistically significant for $p < 0.05$.

Results

The immunosuppressive effects of UVB are mediated by the MC PAFR

The role of MCs in PAFR-mediated systemic immunosuppression was investigated utilizing MC transplants in conjunction with the well-established model of DNFB CHS commonly used to assess immune competence (45–48). The first set of experiments validated a MC transplant model for use in the CHS experiments.

This model made use of bone marrow–derived MCs (BMMCs), which were obtained by flushing bone marrow from femurs and tibia of 8- to 12-wk-old mice and differentiating the cells in culture with IL-3 (10 ng/ml)–supplemented media for 4 wk. Flow cytometry analysis revealed that >90% of the cells were FcεRI⁺c-Kit⁺, suggesting that the cells were mature MCs (Supplemental Fig. 1A). These mature MCs could then be transplanted into the dorsal skin of MC-deficient *Wsh* mice to reconstitute dermal MCs.

WT and *Wsh* mice transplanted with MCs prior to a CHS assay showed that CPAF and UVB irradiation attenuate the ear swelling response to DNFB challenge (i.e., immunosuppression), compared with vehicle-treated mice (Fig. 1B). PAFR KO and *Wsh* mice, however, did not demonstrate CPAF- or UVB-mediated immunosuppression. As expected, MC reconstitution rescued the immunosuppressive phenotype of UVB and CPAF in *Wsh* mice. These findings confirmed that MCs are necessary for UVB/PAFR-mediated inhibition of CHS reactions (19, 49–51).

MC PAFR signaling is required and sufficient for PAFR-mediated immunosuppression

Whereas MCs are necessary for PAFR-mediated immunosuppression, it has not been determined whether MC PAFR is necessary and sufficient for PAF to induce systemic immunosuppression. To test this hypothesis, WT, *Wsh*, or *Wsh* mice transplanted with WT or PAFR KO MCs were treated with either vehicle or CPAF prior to the CHS protocol. Whereas WT MC transplantation to *Wsh* mice rescued the immunosuppressive ability of CPAF, transplantation of PAFR KO MCs failed to rescue this phenotype (Fig. 1C). Moreover, when WT MCs were transplanted into *Wsh* mice lacking PAFR (PAFR KO *Wsh*), CPAF treatment exerted a partial, but still significant, immunosuppressive effect (Fig. 1C). To verify that the partially attenuated response in PAFR KO MC transplantation was not due to poor transplantation yield, the presence of dermal MCs in these mice was demonstrated by toluidine blue staining, and MC numbers were found to be similar to control (Supplemental Fig. 1C). Taken together, these studies indicate that the MC PAFR is both necessary and sufficient to mediate systemic immunosuppression by PAF.

PAF stimulates MC histamine release necessary to induce systemic immunosuppression

MCs are a primary source of the inflammatory mediator histamine. Next, we wanted to determine whether PAFR activation induces MC release of histamine, which is necessary for systemic immunosuppression. It was found in vitro that CPAF induced BMMC production of HDC, the primary synthetic enzyme of histamine, at both the mRNA transcript and protein levels (Fig. 2A, 2B). This coincided with augmented histamine release (Fig. 2C). These findings indicate that MCs are capable of releasing histamine following PAFR activation.

It is known that MC-deficient mice do not exhibit UV-induced immunosuppression, but s.c. injection of histamine elicits an UVB-like immunosuppressive response in WT mice, suggesting that MC-derived histamine may be a mediator in PAF-induced immunosuppression (52). To test this hypothesis, WT, PAFR KO, or *Wsh* mice were treated with vehicle, CPAF, or histamine prior to induction of the CHS response. Unlike CPAF, which only inhibited CHS responses in WT mice, histamine treatment inhibited CHS responses in all mouse strains (Fig. 2D). Furthermore, HDC KO mice, which lack the enzyme responsible for histamine biosynthesis, only exhibited immunosuppression in response to treatment with histamine, but not CPAF (Supplemental Fig. 2A). These data collectively show that the production and

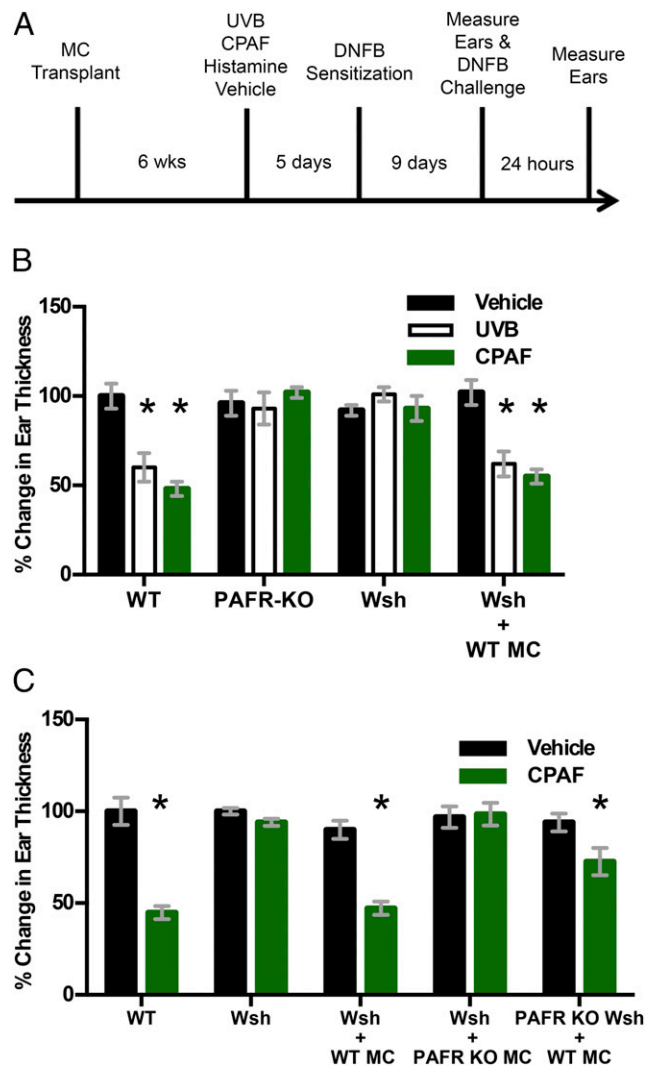


FIGURE 1. The MC PAF-R is necessary and sufficient to attenuate CHS. (A) Model of CHS experiment: MCs (10^6) were injected s.c. into two rows of four injections sites in a 1- by 2-cm area on the shaved dorsal skin of 6- to 8-wk-old *Wsh* or PAFR KO *Wsh* mice. At 6 wk after implantation, mice were used for experiments. Mice were then treated with CPAF (250 ng, i.p.) or vehicle (PBS, i.p.), histamine (200 μ g, s.c.) or UVB (7.5 kJ/m²) on shaved dorsal skin. Five days later mice were sensitized to DNFB by applying 25 μ l of 0.5% DNFB in acetone/olive oil (4:1) on shaved dorsal skin. Nine days later ears were measured; one ear of the mice was treated with 0.5% DNFB, whereas the other ear was treated with vehicle. Ear thickness was measured 24 h later. Change in ear thickness is the difference in ear thickness between the right and left ears. (B) Groups of $n = 8$ –12 WT, PAFR KO, *Wsh*, or *Wsh* mice reconstituted with WT MC were treated with UVB, CPAF, or vehicle 5 d prior to initiation of CHS experiment. (C) Groups of $n = 8$ –9 WT mice, *Wsh* mice, *Wsh* mice reconstituted with WT or PAFR KO MC, or PAFR KO *Wsh* mice reconstituted with WT MC were treated with CPAF or vehicle 5 d prior to initiation of CHS experiment. Data are shown as ear thickness measurements normalized to WT vehicle-treated mice. Results are representative of two separate experiments. Error bars show SEM. * $p < 0.05$, vehicle versus treatment (CPAF or UVB) for each group, determined using a two-way ANOVA and the Sidak post hoc method.

release of histamine are involved in PAF-induced systemic immunosuppression.

To understand whether MC-derived histamine is necessary for PAF-induced immunosuppression, MCs derived from WT or HDC KO mice were transplanted into *Wsh* mice prior to CHS induction and, surprisingly, MC HDC was dispensable for PAF-mediated

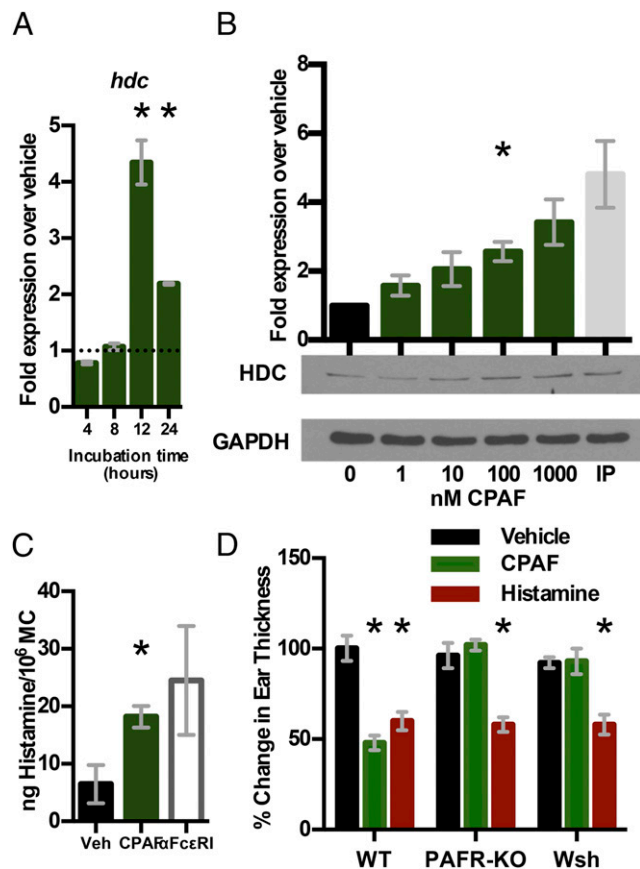


FIGURE 2. MC PAFR activation induces the release of immunosuppressive histamine. **(A)** Relative expression of *hdc* mRNA to *gapdh* was measured by qRT-PCR following treatment of MCs with either vehicle or 100 nM CPAF for 4–24 h. Expression is depicted as fold increase over vehicle following CPAF treatment. Dashed lines represent 1-fold change. Results are representative of three independent experiments. **(B)** Lysates from MCs treated with CPAF or 1 mM ionophore A23187/100 nM PMA for 24 h were immunoblotted for HDC. Quantitation represents mean fold HDC expression over vehicle-treated cells. Blot is representative of three experiments. **(C)** Histamine release in supernatants from cells treated with vehicle, 100 nM CPAF, or 3 μg/ml αFceR Ab for 1 h was measured by EIA. Results depict the mean of three separate experiments. **(D)** Groups of $n = 8$ –12 WT, PAFR-KO, or *Wsh* mice were treated with histamine (200 μg, s.c.), CPAF (250 ng, i.p.), or vehicle 5 d prior to DNFB sensitization. Mice were challenged with DNFB on day 9 postchallenge, and ear thickness was measured 24 h later. Data are shown as ear thickness measurements normalized to vehicle-treated mice. Results are representative of three separate experiments. Error bars represent SEM. * $p < 0.05$, vehicle versus treatment (CPAF or histamine) for each group, determined using a two-way ANOVA (A and D) or one-way ANOVA (B and C) and the Sidak post hoc method (A–C) or the Holm–Sidak method (D), with $\alpha = 5\%$.

immunosuppression (Fig. 3A). However, the transfer of HDC KO MCs into PAFR KO *Wsh* mice that lack both MCs and PAFR did not exhibit CPAF-mediated immunosuppression of the CHS response, whereas the transfer of WT MCs into the PAFR KO *Wsh* mouse facilitated the effect (Fig. 3B). Nontransplanted *Wsh* mice responded appropriately (Supplemental Fig. 2B). These data are consistent with the hypothesis that PAFR signaling in MCs is sufficient to drive immunosuppression via a process involving histamine but that MCs are not necessarily the sole source of PAF-induced histamine.

MC COX-2 is necessary for PAF-induced immunosuppression

Previous work has demonstrated the importance of PGs, specifically PGE₂ acting via its E prostanoïd 4 receptor, in suppression of the

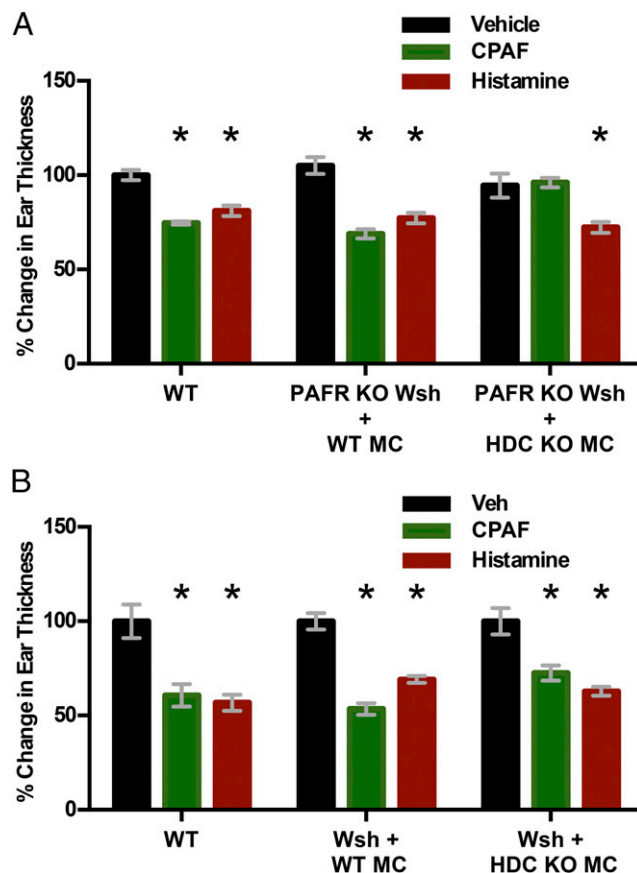


FIGURE 3. MC-derived histamine promotes but is not necessary for PAFR inhibition of CHS response. WT or HDC KO MCs were transplanted into the dorsal skin of *Wsh* **(A)** or PAFR KO/*Wsh* **(B)** mice. Groups of $n = 5$ –8 mice along with WT were treated with vehicle, CPAF i.p., or histamine s.c. 5 d before sensitization to DNFB for CHS assay. Mice were challenged with DNFB on day 9 postchallenge, and ear thickness was measured 24 h later. Data are shown as ear thickness measurements normalized to vehicle-treated mice. **(A)** and **(B)** are representative of two independent experiments each. Error bars represent SEM. * $p < 0.05$, vehicle versus treatment (CPAF or histamine) for each group, determined using a two-way ANOVA and the Holm–Sidak post hoc method, with $\alpha = 5\%$.

immune system by UV light (41). MCs have been documented to synthesize and release PGs and other eicosanoids (21–23), but the role of MC-derived PGs in PAFR-mediated immunosuppression is unknown. We found that CPAF-treatment of BMMCs resulted in increased expression of COX-2 (Fig. 4A, 4B) as well as PGE₂ release (Fig. 4C). As expected, no response was observed when treating BMMCs lacking PAFR (Supplemental Fig. 1C).

PAFR agonist-induced (e.g., UVB, cigarette smoke, and CPAF) immunosuppression can be blocked by COX-2 inhibitors (13, 39, 40, 53). To determine whether this COX-2-dependent immunosuppressive effect was required for the effects of histamine in this system, WT mice were treated with COX-2 inhibitor SC-236 and vehicle, CPAF, or histamine 5 d prior to the CHS protocol. COX-2 inhibitors blocked the immunosuppressive effects of both CPAF and histamine (Fig. 4D). These data are consistent with the hypothesis that COX-2 is involved in mediating the immunosuppressive response of PAF, but possibly downstream of histamine.

To determine whether MC COX-2 expression is required for the immunosuppressive mechanism of PAF, a COX-2-deficient MC model was used. MCs were derived from the bone marrow of *Mcpt5-cre/Ptgs2^{fllox/fllox}* mice for transplantation into *Wsh* mice and subsequent CHS experiments. This well-characterized model

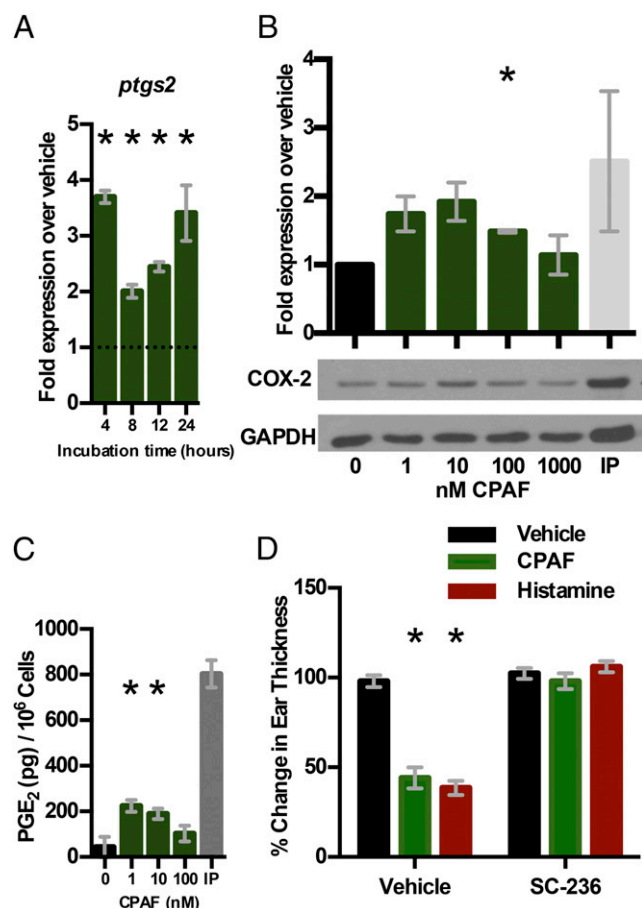


FIGURE 4. COX-2 plays a role in PAFR-mediated immunosuppression and is regulated by PAF and histamine. **(A)** Relative expression of *ptgs2* mRNA to *gapdh* was measured by qRT-PCR following treatment of MCs with either vehicle or 100 nM CPAP for 4–24 h. Expression is depicted as fold increase over vehicle following CPAP treatment. Dashed lines represent 1-fold change. Results are representative of three independent experiments. **(B)** Lysates from MCs treated with CPAP or 1 mM ionophore A23187/100 nM PMA for 24 h were immunoblotted for COX-2 protein. Blot is representative of three experiments. **(C)** PGE₂ release in supernatants from cells treated with vehicle, CPAP, or ionophore/PMA for 24 h were measured by EIA. **(D)** Groups of $n = 6$ –8 WT mice were treated with vehicle or SC-236 (200 ng) and vehicle, histamine (200 μ g, s.c.), or CPAP (250 ng, i.p.) 5 d before sensitization to DNFB for CHS assay. Mice were challenged with DNFB on day 9 postchallenge, and ear thickness was measured 24 h later. Data are shown as ear thickness measurements normalized to vehicle-treated mice. Results are representative of three separate experiments. Error bars represent SEM. * $p < 0.05$, vehicle versus treatment (CPAP or histamine) for each group, determined using a two-way ANOVA (A) or one-way ANOVA (B–D) and the Sidak post hoc method (A and B) or Dunnett method (C and D), with $\alpha = 5\%$.

exploits the MC sp. act. of the *Mcpt5* promoter to generate a cre/ flox conditional KO of the COX-2 (i.e., *Ptgs2*) gene only in MCs (42, 43). BMMCs derived from *Mcpt5-cre/Ptgs2^{flox/flox}* (COX-2 KO) or control *Ptgs2^{flox/flox}* (COX-2 WT) mice were transplanted into *Wsh* mice. These mice and WT controls were treated with vehicle, CPAP, or histamine prior to the CHS protocol. Both histamine and CPAP induced immunosuppression in both WT mice and *Wsh* mice transplanted with COX-WT MCs. In contrast, *Wsh* mice transplanted with COX-2 KO MCs were resistant to CPAP-mediated immunosuppression (Fig. 5). These findings suggest that MC COX-2 contributes to the suppressive effects of PAF on the immune system.

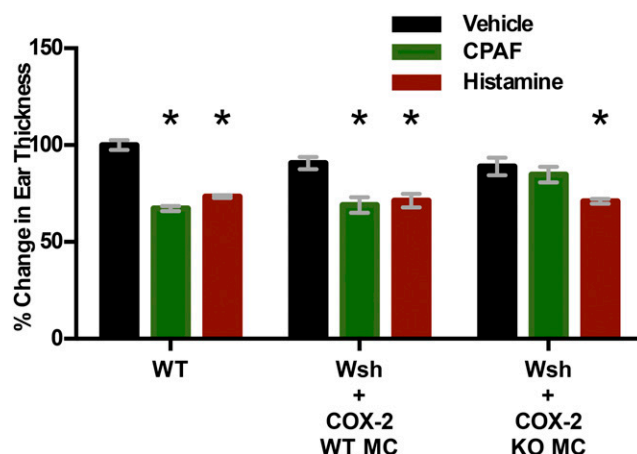


FIGURE 5. MC COX-2 is necessary for PAFR-mediated immunosuppression. WT (*ptgs2^{flox/flox}*) or COX-2 KO (*mcpt5-cre/ptgs2^{flox/flox}*) MCs were transplanted into the dorsal skin of *Wsh* mice. Groups of $n = 7$ –10 mice along with WT were treated with vehicle, CPAP i.p., or histamine s.c. 5 d before sensitization to DNFB for CHS assay. Mice were challenged with DNFB on day 9 postchallenge, and ear thickness was measured 24 h later. Data are shown as ear thickness measurements normalized to vehicle-treated mice. Results are representative of two separate experiments. Error bars represent SEM. * $p < 0.05$, vehicle versus treatment (CPAP or histamine) for each group, determined using a two-way ANOVA and the Holm–Sidak post hoc method, with $\alpha = 5\%$.

PAF-induced MC migration to draining LNs involves COX-2

Recent work in the field has demonstrated that once MCs are activated by UVB or PAF, they migrate to draining LNs, and that this migration is necessary for subsequent immunosuppression (13, 19, 49, 51). Additionally, PGE₂ has been implicated as a mediator of UVB-induced immunosuppression (41). However, the role of PGs in PAFR-induced migration of MCs to draining LNs has not been directly tested. To test whether PGs mediate PAFR-activated MC migration to LNs, *Wsh* mice reconstituted with dermal CD45.1 WT BMMCs were either treated with vehicle or CPAP, and vehicle or the COX-2 inhibitor SC-236. Inguinal LN cells were analyzed for c-Kit⁺/CD45.1⁺ MCs by flow cytometry, revealing reduced MC populations in LNs from mice treated with both CPAP and SC-236 versus CPAP alone, suggesting that COX-2 inhibitors could block the PAFR-mediated migration of MCs to LNs (Fig. 6). Histology revealed similar results (Supplemental Fig. 3). These data are consistent with the hypothesis that MC migration to LNs after PAFR activation is regulated by COX-2 (Fig. 7) (54, 55).

Discussion

PAF and the activation of the MC PAFR have been implicated in mediating the suppressive effects of pro-oxidative stressors on the immune response. These oxidative stressors include UVB, jet fuel, cigarette smoke, photodynamic therapy, and chemotherapy (39, 50, 52, 53, 56). Still, the mechanism of this suppression remains to be fully elucidated. Although the models employed to study these mechanisms have significant precedence in the literature, they have potential limitations to consider. In particular, the use of *Wsh* mice has recently started to lose favor because aberrant c-Kit signaling could cause ambiguous results, and because these mice exhibit abnormal hematopoiesis (57). Moreover, *Wsh* mice have been found to have increased numbers of neutrophils and basophils (58). Although significant, we found that this model is relevant in that most of this work in the field makes use of *Wsh* mice. Furthermore, newer MC-specific models (e.g., *Cpa3-cre*)

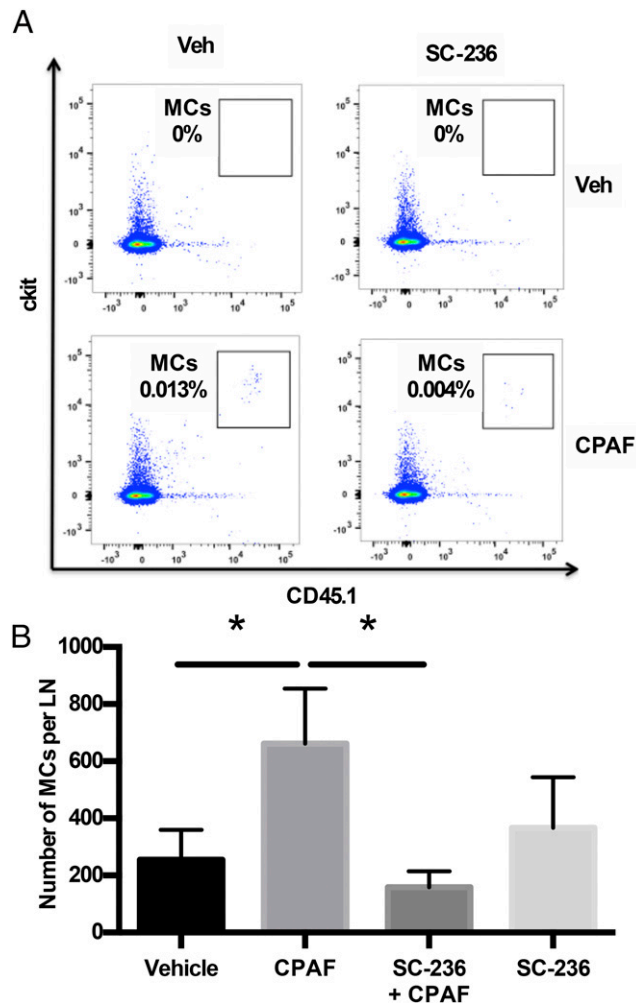


FIGURE 6. SC-236 blocks MC migration to LNs. CD45.1 BoyJ BMMCs (10^6) were transplanted into the dorsal skin of *Wsh* mice. Groups of $n = 3$ mice in each group were then treated with vehicle or CPAF and vehicle or SC-236. Inguinal LNs were harvested 24 h later and filtered to make a single-cell suspension. Cells were stained (CD45.1-PE, c-Kit–allophycocyanin, CD4-PerCP, viability dye) and analyzed by flow cytometry. **(A)** Representative dot plots of c-Kit⁺CD45.1⁺CD4⁻ cells are shown. **(B)** Mean total number of MCs per LN were calculated. The data represent mean $\pm$ SEM of $n = 9$ in each group pooled from three independent experiments. * $p < 0.05$ for comparisons shown by the black bars, determined using one-way ANOVA and the Holm–Sidak post hoc method for multiple corrections, with $\alpha = 5\%$.

also exhibit their own baseline hematopoietic abnormalities (57). There are also reservations with the use of HDC KO mice, as these mice have been reported to have decreased numbers of MCs and also demonstrate abnormal granule formation (59, 60). As a result of the abnormal granule formation, MCs from HDC KO mice may also exhibit abnormal nonhistamine storage and release (60). Nevertheless, we found this model invaluable for investigating the role of MC-derived histamine.

Recent studies have suggested that Cre-expressing cells, even in the absence of a loxP-flanked allele, might generate phenotypes including induction of DNA damage and inhibition of cell growth (61, 62). We have found no evidence that *Mcpt5-cre* expression has an effect on MC function and observed normal MC function with this Cre transgene (63). Thus, although this is a caveat in the interpretation of our experiments, it is unlikely to impact our conclusions. Also related to this point, although we considered using the *Mcpt5-cre/Ptgs2^{fllox/fllox}* mice for initial CHS

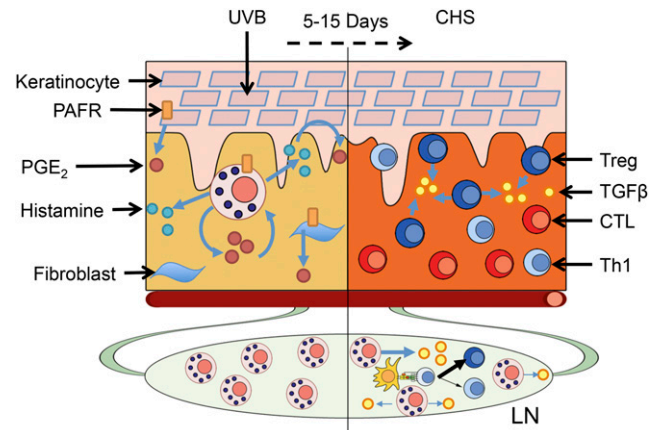


FIGURE 7. Schematic showing proposed mechanism for PAFR-induced immunosuppression. UVB initiates a cascade of events resulting in immunosuppression during the course of 5–15 d for the duration of CHS experiments (for detailed timeline refer to Fig. 1A). UVB irradiation of the skin leads to the formation of PAFR agonists in the skin (20). MC PAFR activation stimulates PGE₂ and histamine release. PAFR activation in keratinocytes and fibroblasts have been shown to induce COX-2 expression (54). Additionally, histamine-stimulated keratinocytes release PGE₂ (55). Subsequently, PGE₂ may possibly activate MCs via autocrine and paracrine mechanisms involving upregulation of chemokine receptor CXCR4 to induce MC migration to draining LNs during the course of 24 h. In the LNs, MCs may induce systemic immunosuppression via production of cytokines such as TGF- β that favor the differentiation of Tregs. This immunosuppression is mediated by Tregs (39), possibly via a decrease in CTL and Th1 populations, resulting in decreased CHS reactions.

experiments, we focused on MC adoptive transfer studies for two reasons. First, mucosal MCs still express COX-2 in *Mcpt5-cre/Ptgs2^{fllox/fllox}* mice, a phenotype that may obscure the role of dermal MC COX-2. Our adoptive transfer system allows us to rescue the immunosuppressive effects of PAF by implanting WT MCs and isolating the role of a particular MC protein by implanting gene-deficient MCs. Second, using this adoptive transfer model across several gene-deficient MC donors allows us to make clearer comparisons between the results of our experiments. These comparisons are an important component of our studies allowing us to accurately define pathways in this model.

Although it seems that the MC PAFR is necessary and sufficient to mount PAFR-mediated systemic immunosuppression, activation of other PAFR-expressing cells (e.g., basophils, keratinocytes, fibroblasts, endothelial cells) may contribute to reaching the threshold necessary to suppress immune responses. The contribution of other PAFR-expressing cells is highlighted by the role of histamine in the immunosuppressive effects of PAF. Histamine appears to be necessary for this mechanism, as mice lacking histamine are impervious to the immunosuppressive effects of PAF (Supplemental Fig. 2). Whereas MCs are the main contributor of histamine in the body, MC-derived histamine seems to be dispensable for PAF-induced immunosuppression (Fig. 3A). This would suggest that other histamine-producing cells are involved in this mechanism, such as basophils, platelets, dendritic cells, T cells, and macrophages. Still, it seems that this second histamine-producing cell requires the presence of PAFR-expressing MCs, as neither *Wsh* or *Wsh* mice engrafted with PAFR KO MCs respond to CPAF (Fig. 1C). Furthermore, these secondary histamine-producing cells also require PAFR expression, as HDC KO MCs rescue the immunosuppressive effects of PAF in *Wsh* but not PAFR KO *Wsh* mice (Fig. 3). This gives rise to two distinct mechanisms for histamine in PAFR-mediated systemic immunosuppression: MC histamine-dependent and

-independent pathways. In the latter, the role of the MC may be to recruit secondary histamine-producing cells either to the skin or to draining LNs to mount the subsequent immunosuppression.

One of the goals of this study was to elucidate the relationship between histamine and COX-2-derived PGs in PAFR-mediated systemic immunosuppression. COX-2 has been shown to be involved in mediating the immunosuppressive effects of UVB and PAF. A proposed mechanism for the involvement of COX-2 in this pathway is the interaction of PGs in the regulation of cell chemotaxis, as PGE₂ has been previously implicated in regulating immune cell chemotaxis (64), and histamine can promote PG release (54, 65). A possible mechanism for the regulation of chemotaxis by PGs is through the regulation of chemokine ligand/receptors. One such example of PGs mediating MC migration is the ability of PGE₂ to induce CXCL12, the CXCR4 ligand, expression in endothelial cells (19). Ullrich and colleagues (19) have shown that MC PAFR activation upregulates CXCR4 expression in MCs and promotes the obligate chemotaxis to draining LNs necessary to mediate immunosuppression. Still, it remains to be elucidated how the CXCR4–CXCL12 chemokine gradient is established to facilitate PAF-induced MC migration to draining LNs.

The present study suggests that COX-2 and histamine mediate the immunosuppressive effects of PAF (see the theoretical model in Fig. 7). It appears that a “proinflammatory” threshold must be reached before subsequent immunosuppression ensues, where COX-2-derived PGs and histamine contribute to the proinflammatory “cytokine storm” activation energy. It would be of particular interest to identify the site of action of histamine and PGs. The present evidence in this study gives rise to two hypotheses for how MCs may be mediating immunosuppression: first, either by direct interactions with lymphocytes in draining LNs following CXCR4-dependent MC migration to LNs, or second, by MC release of mediators in the skin that act locally or travel via lymphatics to affect T cell activation in draining LNs. As mentioned before, MC migration to draining LNs is necessary to mediate systemic immunosuppression, and migration is mediated via CXCR4 chemotaxis following MC PAFR activation (19, 49, 51). It has also been shown that MC-derived particles can travel via lymphatic vessels to signal at distant LNs (66). This could be important because histamine and PGs released following MC PAFR activation could travel to draining LNs to influence T cell activity.

In summary, these studies indicate that systemic immunosuppression induced by PAFR activation is mediated by the MC PAFR. Moreover, MC COX-2 appears to play an important role in this process. Given that systemic PAFR activation is associated with multiple environmental pro-oxidative stressors, from chemotherapy and radiation therapy to cigarette smoking and UVB radiation, an understanding of this process could result in potential therapeutics to address this potentially unwanted immunosuppression.

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Disclosures

The authors have no financial conflicts of interest.

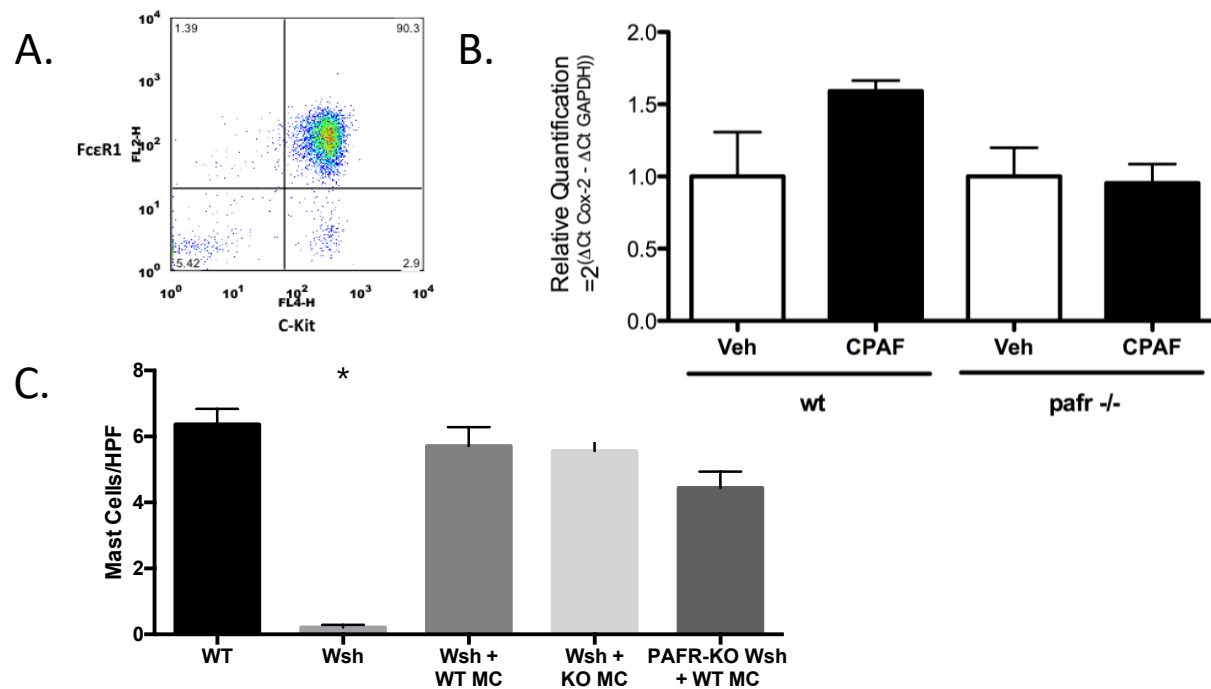
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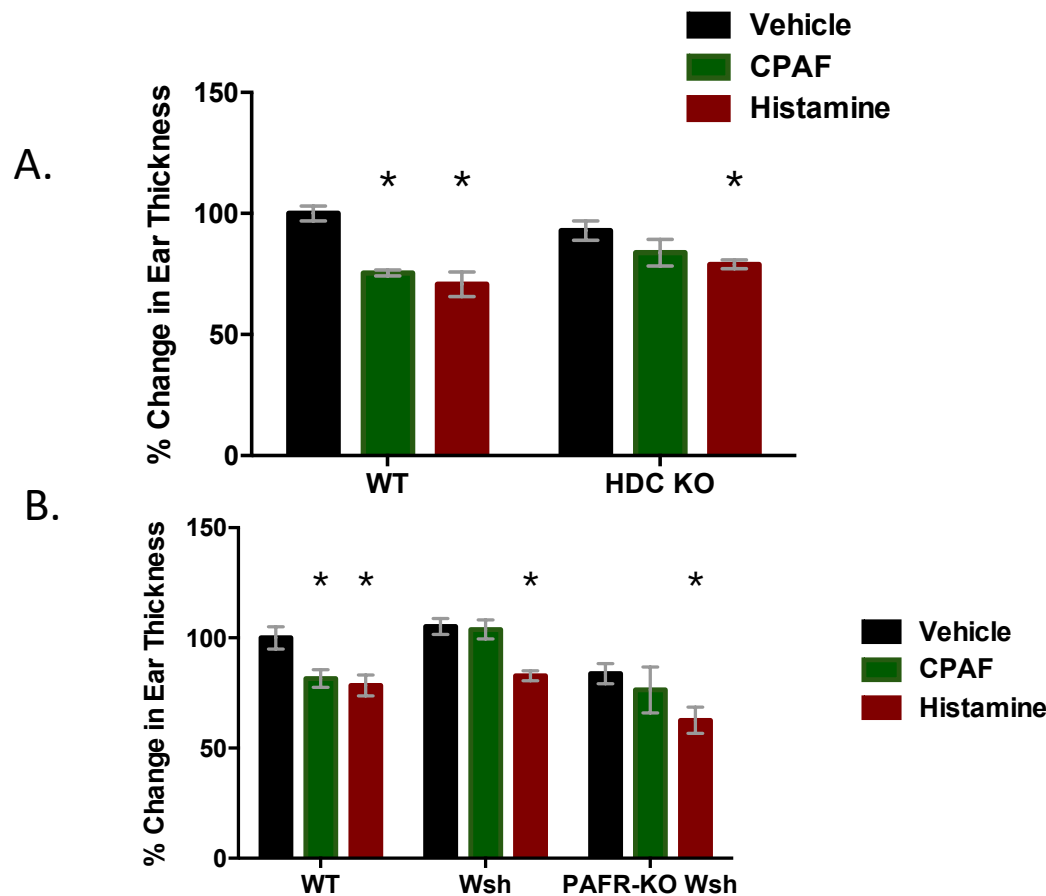
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Supplemental Figures

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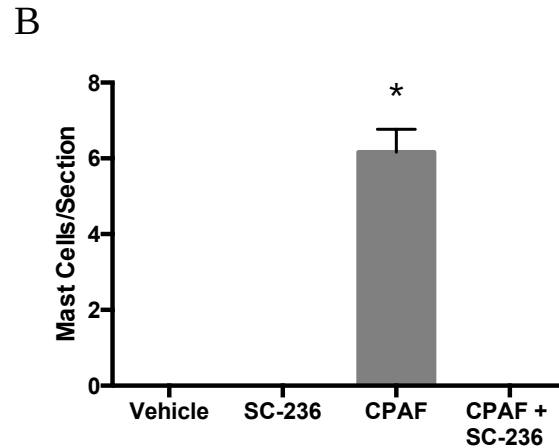
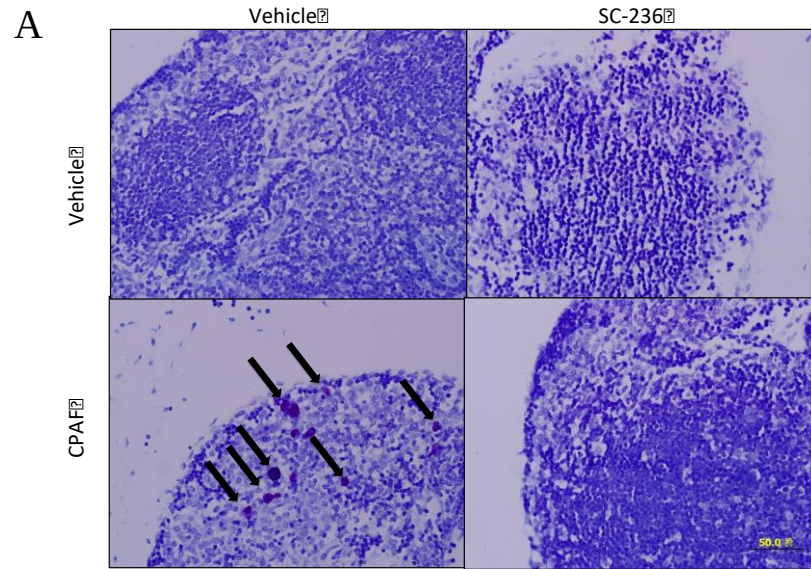


Supplemental Figure 1. BMMC maturation, and *in vitro* and transplant controls. **A.** After four weeks of IL-3 supplementation, BMMCs were analyzed for ckit and FcεRI expression by flow cytometry. Culture method yields >90% ckit⁺ FcεRI⁺ cells. **B.** Relative expression of *ptgs2* mRNA to *gapdh* was measured by qRT-PCR following treatment of wt and pafr^{-/-} MCs with either vehicle, or 100nM CPAF for 4hrs. Expression is depicted as fold increase over vehicle following CPAF treatment. Figure representative of three independent experiments. **C.** Dorsal skin from mice were formalin fixed before storage in ethanol. Sectioned slides were stained for MCs by acidified toluidine blue. MC numbers were quantified by counting ten high power fields (HPF, 600X). Data depicted is the mean of 4 mice ± SEM. * Denotes statistically significant differences (p<0.05) in WT vs. experimental group. Statistical significance determined using one-way ANOVA and the post-hoc Bonferroni method for multiple corrections, with alpha=5%. Error bars represent SEM.



Supplemental Figure 2. A. HDC KO mice are insensitive to CPAF- but sensitive to histamine-induced inhibition of CHS reactions, and B. Effect of CPAF and histamine on CHS reactions in *Wsh* mice.

A. Groups of $n = 10-15$ WT or HDC KO mice were treated with histamine ($200 \mu\text{g s.c.}$), CPAF (250 ng i.p.), or vehicle 5 days prior to DNFB sensitization. Mice were challenged with DNFB on Day 9 post-challenge and ear thickness was measured 24 hours later. Data shown as ear thickness measurements normalized to vehicle treated mice. Figure is representative of three separate experiments. **B.** Groups of $n = 4-8$ WT, *Wsh*, or PAFR KO *Wsh* mice were treated with histamine ($200 \mu\text{g s.c.}$), CPAF (250 ng i.p.), or vehicle 5 days prior to DNFB sensitization. Mice were challenged with DNFB on Day 9 post-challenge and ear thickness was measured 24 hours later. Data shown as ear thickness measurements normalized to vehicle treated mice. Figure is representative of three separate experiments. * Denotes statistically significant differences ($p < 0.05$) in Vehicle vs. treatment (CPAF or histamine) for each group. Statistical significance determined using two-way ANOVA and the post-hoc Holm-Sidak method, with $\alpha = 5\%$. Error bars represent SEM.



Supplemental Figure 3. Histological analysis of PAF-induced MC migration to LNs blocked by COX-2 inhibitors.

A. CD45.1 BOYJ BMMCs (10^6) were transplanted into the dorsal skin of Wsh mice. Groups of 2-3 mice were then treated with vehicle or CPAF, and vehicle or SC-236. Inguinal LNs were harvested 24hrs later, formalin fixed, sectioned and stained by toluidine blue. Arrows point at MCs. Images were taken at 400X. Black bar represents 50 μ m. **B.** MC numbers were quantified using by counting all the MCs per slide and dividing by the number of LNs on the slide. Data depicted is the mean $\pm$ SEM of three slides from each mouse. Error bars represent SEM. * Denotes statistically significant differences ($p < 0.05$) in vehicle vs. CPAF.