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Platelet-activating factor-induced reduction in contact hypersensitivity responses is mediated by mast cells via cyclooxygenase-2-dependent mechanisms

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Abstract

Platelet-activating factor (PAF) stimulates numerous cell types via the activation of the G-protein coupled PAF-receptor (PAFR). PAFR activation not only induces acute pro-inflammatory responses, but also delayed systemic immunosuppressive effects by modulating host immunity. While 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 mechanism(s), and the 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 prostaglandin E₂ (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 prostaglandins 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.

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Keywords

Mast cells; cyclooxygenase; Platelet-activating factor; histamine; histidine decarboxylase

INTRODUCTION

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, PAFR (*Ptafi*), expressed in many cell types, including: platelets, neutrophils, macrophages, dendritic cells, and mast cells (MC) (8–11). PAF stimulates the activation of platelets (12–14), induces macrophage, neutrophil and eosinophil chemoattraction (15–17), neutrophil extracellular trap release (18), and it has recently been discovered to stimulate MC migration to lymph nodes (LN) (19). While the pro-inflammatory 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. Like 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, and 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 pro-inflammatory role in many immune responses.

In contrast to these pro-inflammatory 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 Treg differentiation via TGF β -dependent mechanisms when co-cultured with naïve T cells (30), and augment the suppressor activity of myeloid-derived suppressor cells (MDSCs) (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 anti-tumor immunity to promote skin tumorigenesis (35, 36), and mobilize Tregs and MDSCs in the tumor microenvironment (37).

MCs mediate the immunosuppressive effects of UV light, and subcutaneously 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 prostanoid 4 (EP4) 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 prostaglandins may be relevant to the immunosuppressive mechanisms of PAF. The current studies set out to further delineate the link between PAF, MCs, histamine, and COX-2 generated prostaglandin 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) and TGF- β inhibitor (LY364947), and histamine EIA kit were obtained from Cayman Chemicals (Ann Arbor, MI). PGE₂ EIA was obtained from R&D (Minneapolis, MN). qPCR reagents were obtained from Invitrogen (Carlsbad, CA). Primers for qPCR were obtained from IDT (Coralville, IA). Antibodies for immunoblot analysis were obtained from Abcam (Cambridge, MA; histidine decarboxylase (HDC): Rabbit polyclonal #ab37291; COX-2: Rabbit monoclonal #ab62331). Mast cells 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 weeks. Cell cultures contained MC populations (Fc ϵ R⁺, c-kit⁺) greater than 90% as measured by flow cytometry, using antibodies 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 of IL-3 in 12 well plates.

Mice

C57BL/6 (WT; Charles River Laboratories, Wilmington, MA) and C57BL/6 *Kit^{Wsh/Wsh}* (*Wsh*) (B6.Cg-Kit^{W-sh}/HNhrJaeBsmGlljJ, The Jackson Laboratory, Bar Harbor, ME) mice were obtained commercially. *Ptafr*^{-/-} (PAFR 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^{flox/flox}* and *Ptgs2^{flox/flox}* 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 6–14 week 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^{flox/flox}* and *Ptgs2^{flox/flox}* mice, the University of Pennsylvania. All procedures were approved by the Animal Care and Use Committees of respective institutions.

Mast cell transplantation and contact hypersensitivity assay

MCs (10⁶) were injected subcutaneously into two rows of four injection sites in a 1 by 2 cm area on the shaved dorsal skin of 6–8 wk old *Wsh* or PAFR KO *Wsh* mice. After 6 weeks post-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 μg s.c.), or CPAF (200 ng i.p.). Five days post-treatment 25 μL of 0.5% 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, $\text{mm} \times 10^{-2}$). Subsequently, one ear was treated with 10 μL of 0.5% DNFB while the other ear was treated with vehicle. After 24 hours, 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 Figure 1A for details).

Histology

Dorsal skin samples or LNs from mice were formalin fixed for 24 hours before storage in ethanol. Specimens were paraffin embedded, sectioned and stained for MCs using acidified toluidine blue by the IUSM Histology Core (44). MC numbers were quantified by counting ten high power fields (HPF, 600 \times).

qRT PCR

Total RNA was extracted from treated MCs using the RNeasy kit (Qiagen). In brief, tissue was homogenized in RLT buffer containing 2-mercaptoethanol 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 was performed for *Ptgs2* and *Hdc* against *Gapdh* as the endogenous control using the Ct method on a Step One Real-Time PCR machine (Applied Biosystems, Foster City, CA). Each assay was performed in triplicate in a 10 μL reaction volume with Taqman Master Mix (SA Biosciences, Frederick, MD), 1 ng cDNA, primers at 500 nM and probe at 250 nM.

Flow cytometry

Cells (10^6) were taken from culture and plated in a 96-well plate in FACS buffer (PBS, 1% BSA, 0.1% NaN_3) for staining. Cells were incubated with Fc Block (BD #553142) for 15 minutes and then stained with 1:100 or 1:200 dilutions of conjugated primary antibody (CD45.1-PE, ckit-APC, CD4-PerCP, Fc ϵ R-PE) for 30 minutes. 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 about 10^5 cells/100 μL for analysis on the Invitrogen Attune Cytometer or the BD LSR II.

Western Blotting and Densitometry

Cells (6×10^6) were harvested and washed twice with PBS. Cell pellets were then lysed with 150 μL of lysis buffer (1% Triton-X, 10 mM Tris base, 150 mM NaCl, 1% protease inhibitor mixture (Sigma); pH 7.4) and centrifuged for 15 minutes at $400 \times g$ at 4°C . Supernatants were collected (120 μL), and the protein concentration was measured by BioRad spectrophotometry methods. Protein (30–100 μg) with β -mercaptoethanol-containing reducing SDS sample buffer was then loaded into a 10% acrylamide gel and run for 45 minutes under a constant 400 mAmps. Proteins were then transferred onto a nitrocellulose membrane for 1 hour at a constant 100 volts at 4°C . The membrane was then cut and

incubated with blocking buffer (5% powdered milk, 0.1% Tween-20, in PBS) for 2 hrs at room temperature (RT) or overnight at 4°C. Membranes were then incubated with the appropriate amount of antibody for 2 hr 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 minutes each before incubation with HRP-conjugated secondary antibody (1:5000) for 30 min at RT. Membranes were then washed with TBST four times for 5 minutes per wash before incubating the membranes with HRP-substrate (Millipore, Billerica, MA) for 1 minute. 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 gene of interest/GAPDH pixel density) normalized to vehicle ratio.

ELISA

Cells (3×10^6) were plated and treated for the described length of time. Supernatants were then collected from suspensions centrifuged at $400 \times g$ for 10 minutes. These supernatants were then diluted according to the detectable range of the ELISA (1:10 or 1:100) in media or analysis buffer.

Statistics

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 when the *p* value was less than 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 dinitrofluorobenzene (DNFB) contact hypersensitivity (CHS) commonly utilized to assess immune competence (45–48). The first set of experiments validated a MC transplant model for the use in the CHS experiments. This model made use of bone marrow-derived mast cells (BMMCs), which were obtained by flushing bone marrow from femurs and tibia of 8–12-week-old mice and differentiating the cells in culture with IL-3 (10 ng/mL) supplemented media for 4 weeks. Flow cytometry analysis revealed that >90% of the cells were FcεRI⁺ c-Kit⁺, suggesting that the cells were mature MCs (Supplemental Figure 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 to vehicle treated mice (Figure 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

While MCs are necessary for PAFR-mediated immune suppression, it has not been determined if 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. While WT MC transplantation to *Wsh* mice rescued the immunosuppressive ability of CPAF, transplantation of PAFR KO MCs failed to rescue this phenotype (Figure 1C). Moreover, if WT MCs were transplanted into *Wsh* mice lacking PAFR (PAFR KO *Wsh*), CPAF treatment exerted a partial, but still significant immunosuppressive effect (Figure 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 Figure 1C). 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 if PAFR activation induces MC release of histamine, which is necessary for systemic immunosuppression. It was found *in vitro* that CPAF induced BMDC production of histidine decarboxylase, the primary synthetic enzyme of histamine, at both the mRNA transcript and protein level (Figure 2A and 2B). This coincided with augmented histamine release (Figure 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 subcutaneous 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 the mouse strains, (Figure 2D). Furthermore, histidine decarboxylase KO (HDC KO) mice, which lack the enzyme responsible for histamine biosynthesis, only exhibited immunosuppression in response to treatment with histamine, but not CPAF (Supplemental Figure 2A). These data collectively show that the production and 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 immunosuppression (Figure 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, while the transfer of WT MCs into the PAFR KO *Wsh* mouse facilitated the effect (Figure 3B). Non-transplanted *Wsh* mice responded appropriately (Supplemental Figure 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 prostaglandins, specifically PGE₂ acting via its EP4 receptor, in suppression of the immune system by UV light (41). MCs have been documented to synthesize and release prostaglandins and other eicosanoids (21–23), but the role of MC-derived prostaglandins in PAFR-mediated immunosuppression is unknown. We found that CPAF-treatment of BMMCs resulted in increased expression of COX-2 (Figure 4A, B), and PGE₂ release (Figure 4C). As expected, no response was observed when treating BMMCs lacking PAFR (Supplemental Figure 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 if 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 days prior to the CHS protocol. COX-2 inhibitors blocked the immunosuppressive effects of both CPAF and histamine (Figure 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 if MC COX-2 expression is required for the immunosuppressive mechanism of PAF, a COX-2 deficient MC model was utilized. MCs were derived from the BM of *Mcpt5-cre/Ptgs2^{flox/flox}* mice for transplantation into *Wsh* mice and subsequent CHS experiments. This well-characterized model exploits the MC-specific activity of the *Mcpt5* promoter to generate a cre/flox conditional knock-out 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, CPAF, or histamine, prior to the CHS protocol. Both histamine and CPAF 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 CPAF-mediated immunosuppression (Figure 5). These findings suggest that MC COX-2 contributes to the suppressive effects of PAF on the immune system.

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). Yet, the role of prostaglandins in PAFR-induced migration of MCs to draining LNs has not been directly tested. To test if prostaglandins mediate PAFR-activated MC migration to LNs, *Wsh* mice reconstituted with dermal CD45.1 WT BMMCs were either treated with vehicle or CPAF, and vehicle or 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 CPAF and SC-236 versus CPAF alone, suggesting that COX-2 inhibitors could block the PAFR-mediated

migration of MCs to LNs (Figure 6). Histology revealed similar results (Supplemental Figure 3). These data are consistent with the hypothesis that MC migration to LNs after PAFR activation is regulated by COX-2.

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, 52–54). Still, the mechanism of this suppression remains to be fully elucidated. While 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 due to the fact that aberrant c-kit signaling could ambiguate results, and that these mice exhibit abnormal hematopoiesis (55). Moreover, Wsh mice have been found to have increased numbers of neutrophils and basophils (56). While 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) also exhibit their own baseline hematopoietic abnormalities (55). 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 (57, 58). As a result of the abnormal granule formation, MCs from HDC KO mice may also exhibit abnormal non-histamine storage and release (58). 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 (59, 60). We have found no evidence that *Mcpt5-cre* expression has an effect on MC function and observe normal mast cell function with this Cre transgene (61). Thus, while this is a caveat in the interpretation of our experiments, it is unlikely to impact our conclusions. Also related to this point, while we considered using the *Mcpt5-cre/Ptgs2^{fllox/fllox}* mice for initial CHS 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 which 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. Secondly, using this adoptive transfer model across several gene-deficient mast cell 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.

While 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 Figure 2). While MCs are the main contributor of histamine in the body, MC-derived histamine seems to be dispensable for

PAF-induced immunosuppression (Figure 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 (Figure 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 (Figure 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 lymph nodes to mount the subsequent immunosuppression.

One of the goals of this study was to elucidate the relationship between histamine and COX-2 derived prostaglandins 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 prostaglandins in the regulation of cell chemotaxis, as PGE₂ has been previously implicated in regulating immune cell chemotaxis (62), and histamine can promote prostaglandin release (63, 64). A possible mechanism for the regulation of chemotaxis by prostaglandins is through the regulation of chemokine ligand/receptors. One such example of prostaglandins mediating MC migration is the ability of PGE₂ to induce CXCL12, the CXCR4 ligand, expression in endothelial cells (19). The Ullrich laboratory has shown that MC PAFR activation upregulates CXCR4 expression in MCs and promotes the obligate chemotaxis to draining LNs necessary to mediate immunosuppression (19). Still, it remains to be elucidated how the CXCR4-CXCL12 chemokine gradient is established to facilitate PAF-induced MC migration to draining LNs.

The work presented herein suggests that COX-2 and histamine mediate the immunosuppressive effects of PAF (Theoretical Model Figure 7). It appears that a “pro-inflammatory” threshold must be reached before subsequent immunosuppression ensues, where COX-2-derived prostaglandins and histamine contribute to the pro-inflammatory “cytokine storm” activation energy. It would be of particular interest to identify the site of action of histamine and prostaglandins. The current evidence in this work gives rise to two hypotheses for how MCs may be mediating immune suppression: 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 (65). This could be important because histamine and prostaglandins 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

CHS	contact hypersensitivity
COX-2	cyclooxygenase type 2
CPAF	1-hexadecyl-2-N-methylcarbamoyl glycerophosphocholine
DNFB	dinitrofluorobenzene
GPC	glycerophosphocholine
Ox-GPC	oxidized GPC
IL-10	interleukin 10
PAF	platelet-activating factor
PAFR	PAF receptor
ROS	reactive oxygen species

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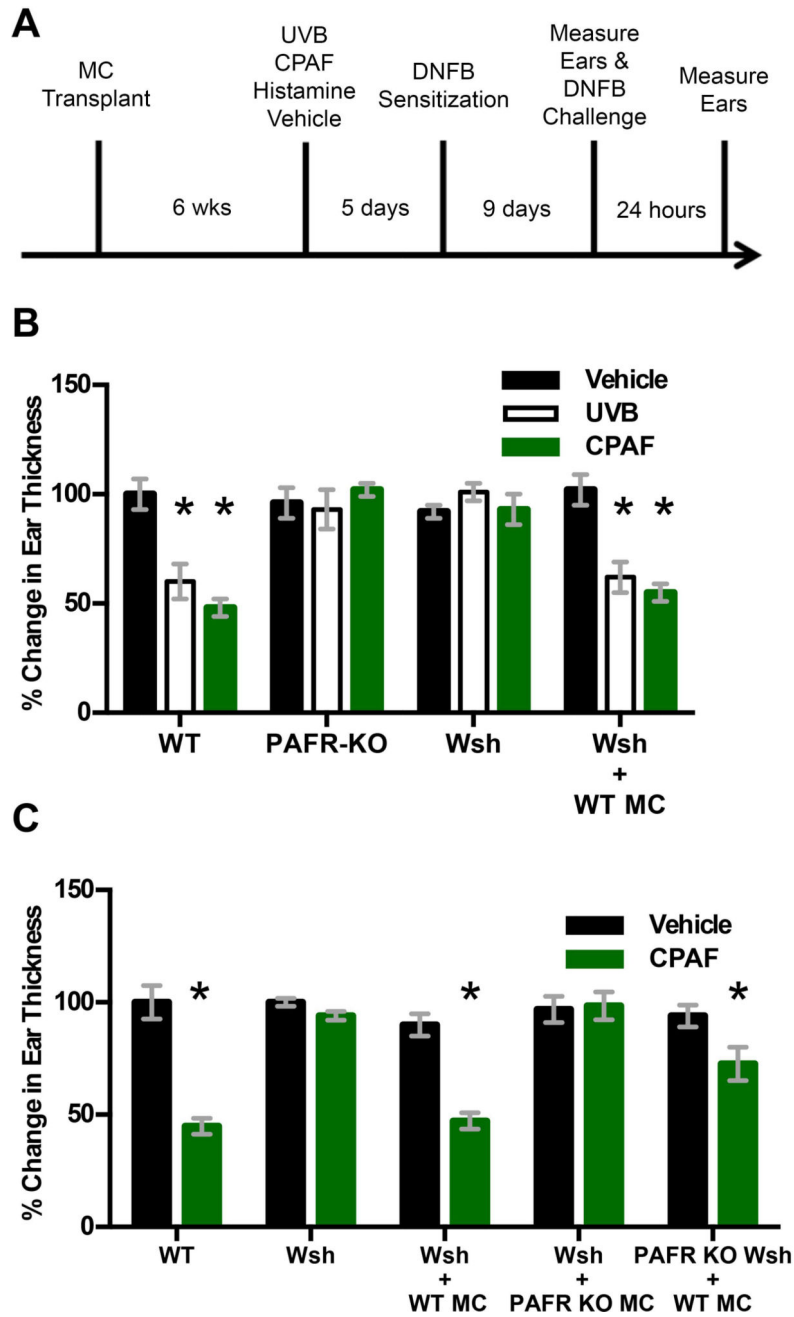


Figure 1. The MC PAF-R is necessary and sufficient to attenuate CHS

A. Model of CHS experiment: MCs (10^6) were injected subcutaneously into two rows of four injections sites in a 1 by 2 cm area on the shaved dorsal skin of 6–8 wk old *Wsh* or PAFR KO *Wsh* mice. After 6 weeks post-implantation, mice were used for experiments. Mice were then treated with CPAF (250ng, i.p.) or vehicle (PBS, i.p.), histamine (200 μ g, s.c.) or UVB (7.5kJ/m²) on shaved dorsal skin. Five days later mice are 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 are measured, one ear of the mice was treated with 0.5% DNFB, whereas the other was treated with vehicle. Ear thickness was measured 24 hours later. Change in ear thickness

is the difference in ear thickness between 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 days prior to initiation of CHS experiment. **C.** Groups of n= 8–9 WT, *Wsh*, *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 days prior to initiation of CHS experiment. Data shown as ear thickness measurements normalized to WT vehicle treated mice. Figure representative of two separate experiments. * Denotes statistically significant differences ($p < 0.05$) in Vehicle vs. treatment (CPAF or UVB) for each group. Statistical significance was determined using two-way ANOVA and the post-hoc Sidak's method. Error bars show SEM.

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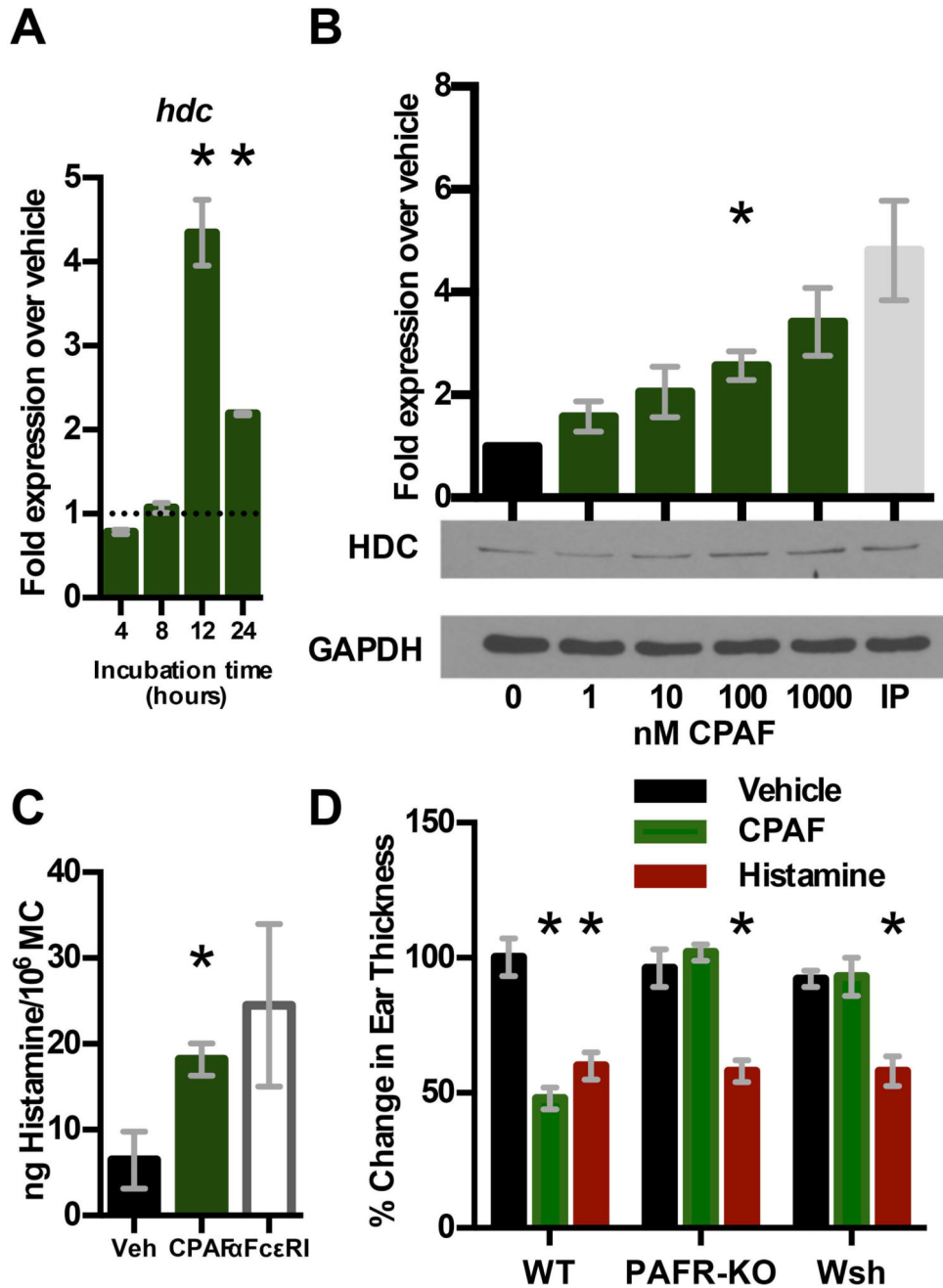


Figure 2. MC PAFR activation induces the release of immunosuppressive histamine
A.) Relative expression of *hdc* mRNA to *gapdh* was measured by qRT-PCR following treatment of MCs with either vehicle, or 100nM CPAF for 4–24hrs. Expression is depicted as fold increase over vehicle following CPAF treatment. Dashed line represent 1-fold change. Figure representative of three independent experiments. **B.)** Lysates from MCs treated with CPAF or 1 mM ionophore A23187/100 nM PMA for 24 hours 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, 100nM CPAF or 3µg/ml αFcεR Ab for 1 hour

was measured by EIA. Figure depicts the mean of three separate experiments. **D.**) Groups of n= 8–12 WT, PAF-R KO or *Wsh* mice were treated with histamine (200 μg 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 (A and D) or one-way ANOVA (B and C) and the post-hoc Sidak (A–C) or Holm-Sidak (D) method, with $\alpha = 5\%$. Error bars represent SEM.

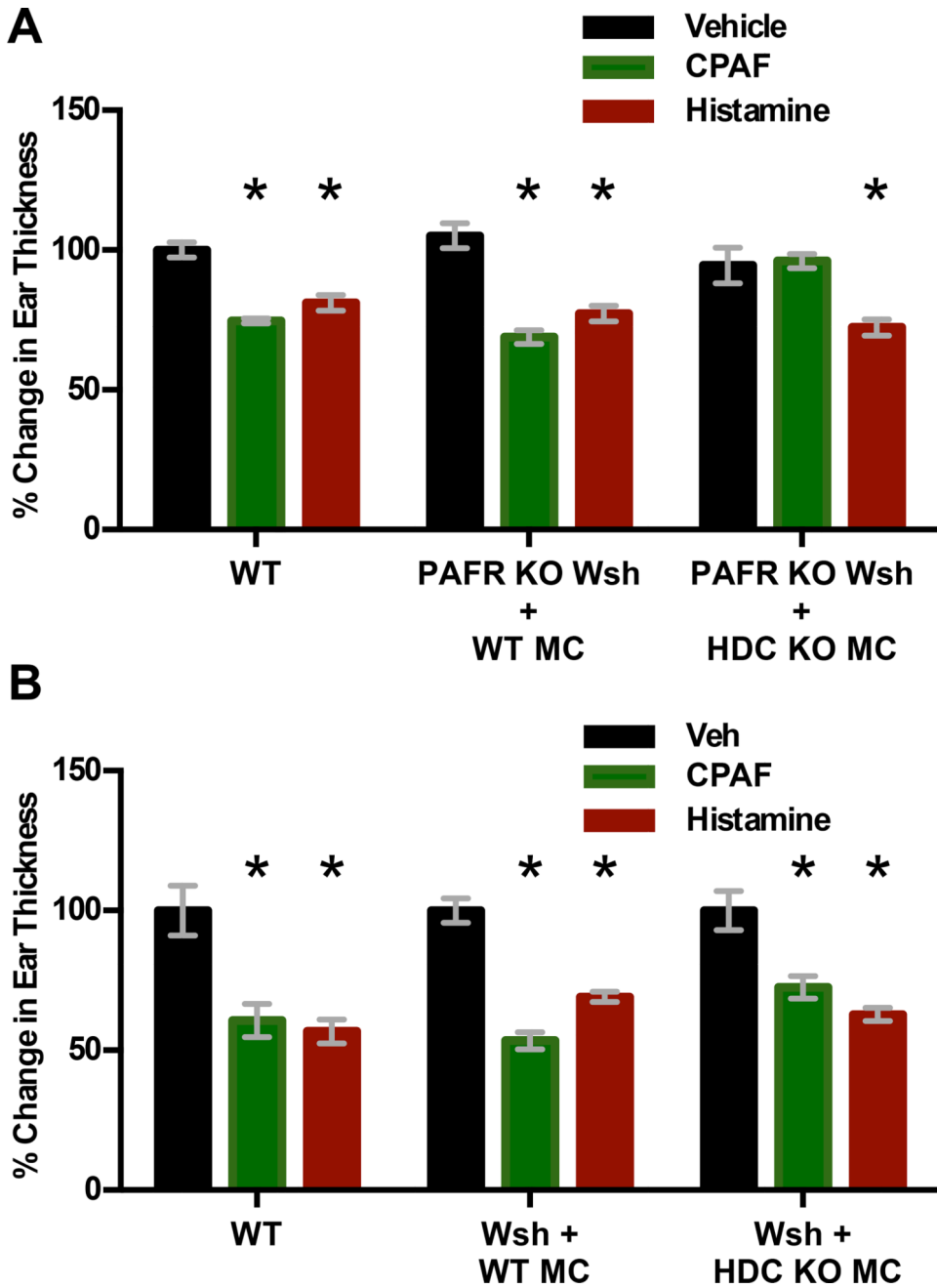


Figure 3. MC-derived histamine promotes but is not necessary for PAFR inhibition of CHS response
 WT or HDC KO MCs were transplanted into the dorsal skin of *Wsh* (A) or PAFR KO/*Wsh* (B) mice. Groups of n= 5–8 mice along with WT were treated with vehicle, CPAF i.p. or histamine s.c. 5 days before sensitization to DNFB for CHS assay. 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. Figures A and B representative of two independent experiments each. * 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.

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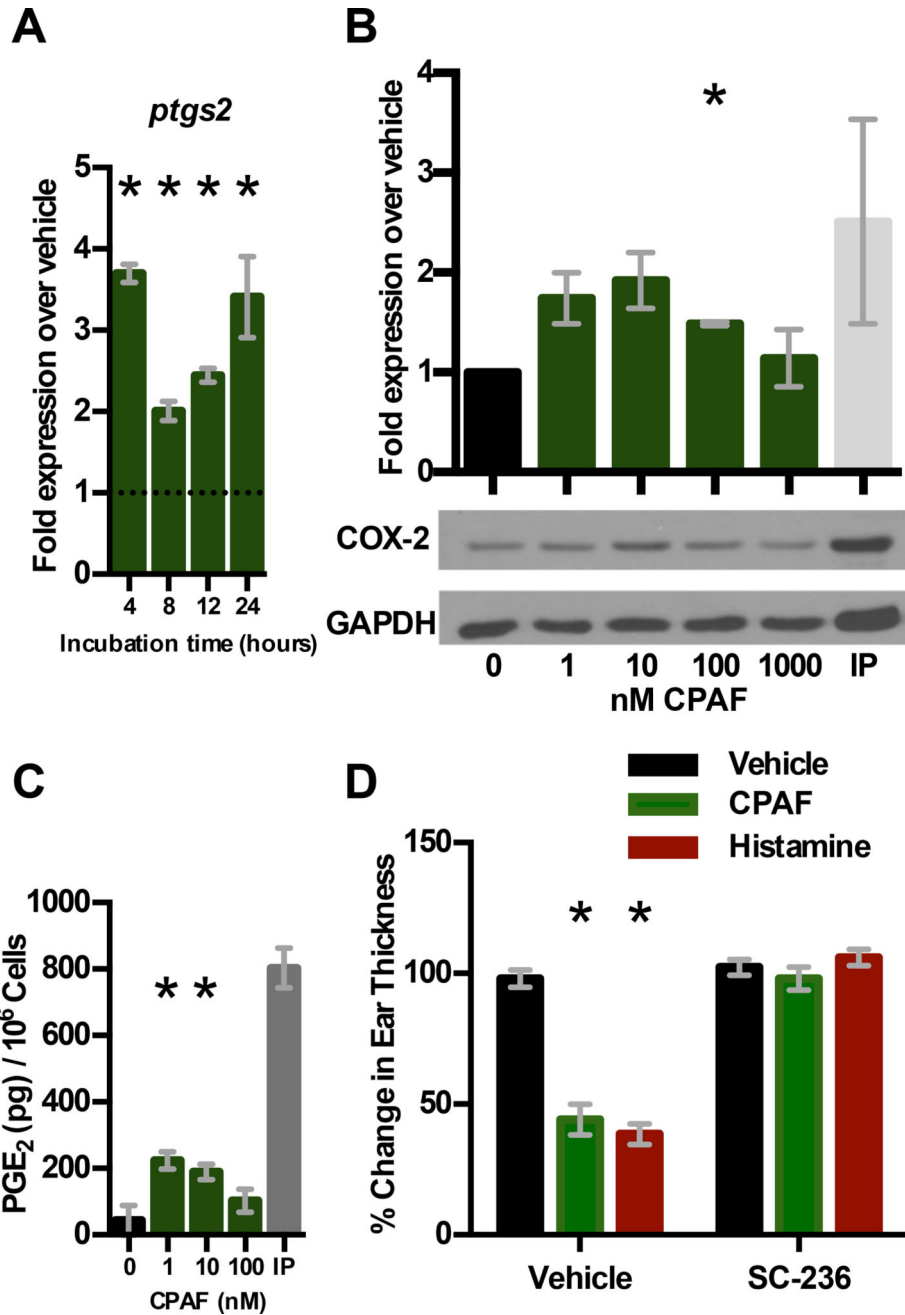


Figure 4. COX-2 plays a role in PAFR-mediated immunosuppression and is regulated by PAF and histamine

A.) Relative expression of *ptgs2* mRNA to *gapdh* was measured by qRT-PCR following treatment of MCs with either vehicle, or 100nM CPAF for 4–24hrs. Expression is depicted as fold increase over vehicle following CPAF treatment. Dashed lines represent 1-fold change. Figure representative of three independent experiments. B.) Lysates from MCs treated with CPAF or 1 mM ionophore A23187/100 nM PMA for 24 hours were immunoblotted for COX-2 protein. Quantitation represents fold COX-2 expression over vehicle-treated cells. Blot is representative of three experiments. C.) PGE₂ release in

supernatants from cells treated with vehicle, CPAF or Ionophore/PMA for 24hrs 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 µg s.c.), or CPAF (250 ng i.p.) 5 days before sensitization to DNFB for CHS assay. 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 (A) or one-way ANOVA (B-D) and the post-hoc Sidak (A and B) or Dunnett's (C and D) method, with alpha=5%. Error bars represent SEM.

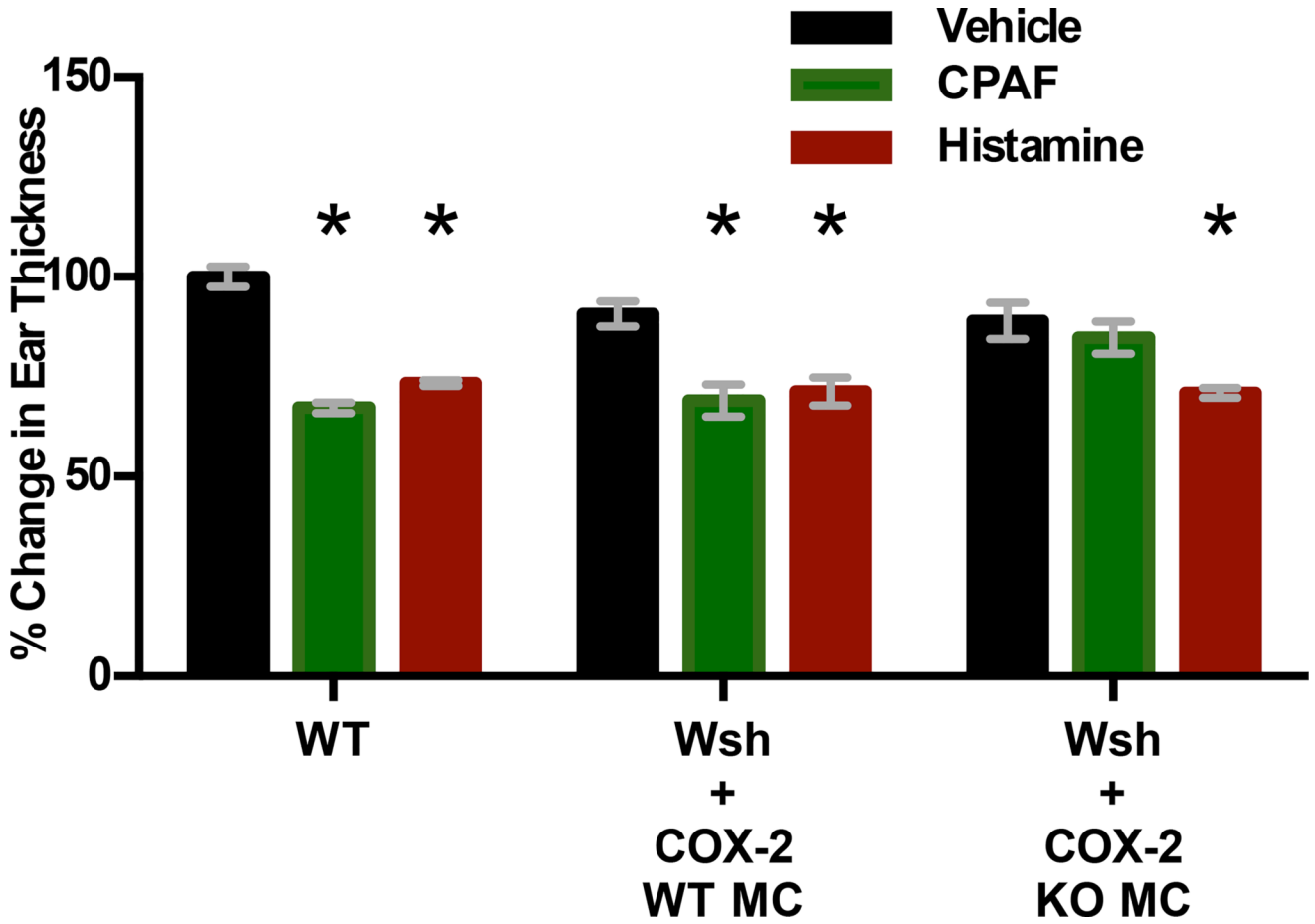


Figure 5. MC COX-2 is necessary for PAFR mediated immunosuppression

WT (*ptgs2^{fllox/fllox}*) or COX-2 KO (*mcpt5-cre/ptgs2^{fllox/fllox}*) MCs were transplanted into the dorsal skin of *Wsh* mice. Groups of n= 7–10 mice along with WT were treated with vehicle, CPAF i.p. or histamine s.c. 5 days before sensitization to DNFB for CHS assay. 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 representative of two separate experiments. * Denotes statistically significant differences (p<0.05) in 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.

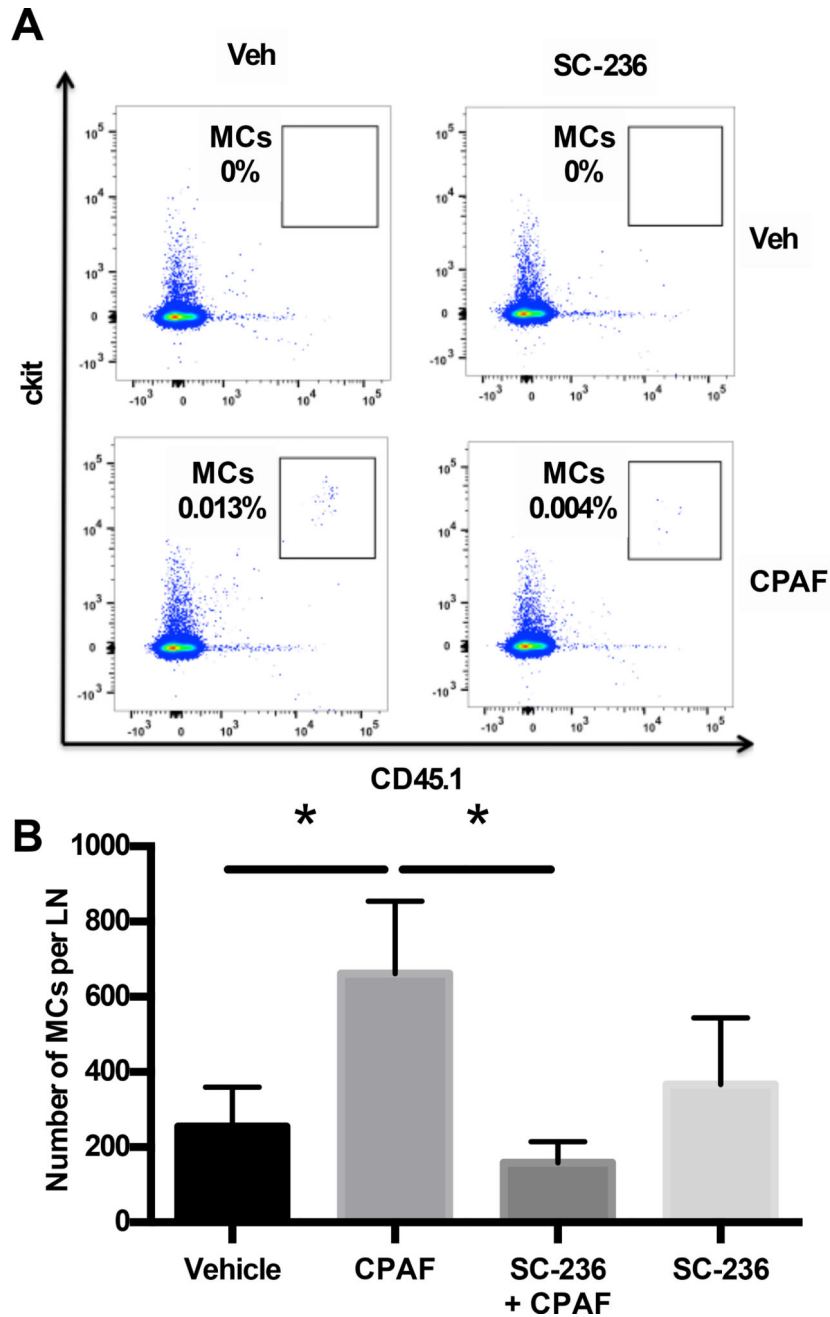


Figure 6. SC-236 blocks MC migration to LNs

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 hrs later and filtered to make a single cell suspension. Cells were stained (CD45.1-PE, ckit-APC, CD4-PerCP, viability) and analyzed by flow cytometry.

A. Figure depicts representative dot plots of ckit+ CD45.1+ CD4- cells. **B.** Mean total number of MCs per LN were calculated. * Denotes statistically significant differences ($p < 0.05$) in the comparisons shown by the black bars. The data represent mean \pm SEM of $n = 9$ in each group pooled from three independent experiments. Statistical significance

determined using one-way ANOVA and the post-hoc Holm-Sidak method for multiple corrections, with alpha=5%.

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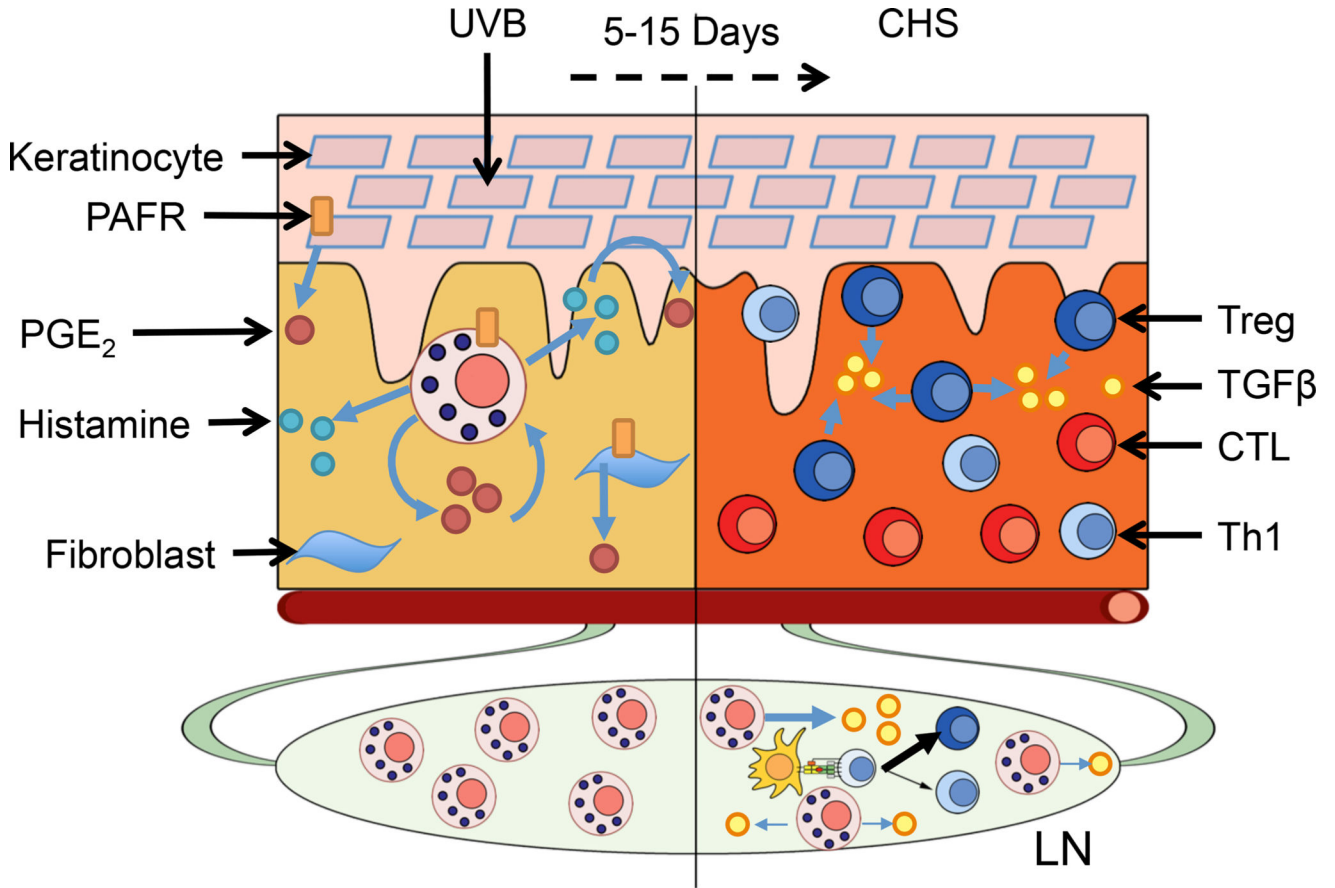


Figure 7. Schematic showing proposed mechanism for PAF-R-induced immunosuppression UVB initiates a cascade of events resulting in immunosuppression over the course of 5–15 days for the duration of CHS experiments (for detailed timeline refer to Fig 1A). UVB irradiation of the skin leads to the formation 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 (66). Additionally, histamine-stimulated keratinocytes release PGE₂ (64). Subsequently, PGE₂ may possibly activate MCs via autocrine and paracrine mechanisms involving upregulation of chemokine receptor CXCR4 to induce MC migration to draining lymph nodes over the course of 24 hours. In the LNs, MCs may induce systemic immunosuppression via production of cytokines like TGFβ 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.