MAST CELLS MEDIATE SYSTEMIC IMMUNOSUPPRESSION INDUCED BY PLATELET-ACTIVATING FACTOR VIA HISTAMINE AND CYCLOOXYGENASE-2 DEPENDENT MECHANISMS

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Dedication

JMJ

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Jesus A. Ocaña

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Platelet-activating Factor (PAF) stimulates various cell types by the activation of the G-protein coupled PAF-receptor (PAFR). Systemic PAFR activation induces an acute pro-inflammatory response, as well as delayed systemic immunosuppressive effects in vivo. De novo enzymatic PAF synthesis and degradation are closely regulated, but oxidative stressors, such as UVB, and cigarette smoke, can generate PAF-like species via the oxidation of membrane lipids in an unregulated process. Mast cells (MCs) and the PAFR have been shown to be necessary to mediate the resulting systemic immune suppression from oxidative stressors. The work herein implicates pro-oxidative chemotherapeutics, such as melphalan and etoposide, in mediating augmentation in tumor growth by inducing the generation of PAFR agonists via the oxidation of membrane lipids. This work also demonstrates the role of MCs and MC-released mediators in PAFR systemic immunosuppression. Through a contact hypersensitivity (CHS) model, the MC PAFR was found to be necessary and sufficient for PAF to mediate systemic immunosuppression. Additionally, activation of the MC PAFR seems to induce MC histamine and prostaglandin E2 release. Furthermore, by transplanting histamine- or COX-2-deficient MCs into MC-deficient mice, MC-derived histamine and prostaglandin release were found to be necessary for PAF to induce systemic immunosuppression.

Lastly, we have evidence to suggest that prostaglandin release modulates MC migration to draining lymph nodes, a process necessary to promote immunosuppression. These studies fit with the hypothesis that MC PAFR activation mediates PAFR systemic immunosuppression in part by histamine and prostaglandin release.

Ahmad Safa, Ph.D., Chair

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List of Abbreviations

5-LO	5-Lipooxygenase
AC	Adenylyl cyclase
AcCoA	Acetyl Coenzyme A
ADAM	A disintegrin and metalloprotease
AMP	adenosyl monophosphate
APC	antigen presenting cells
BMMC	Bone marrow-derived mast cell
cAMP	Cyclic adenosyl monophosphate
CD	Cluster of differentiation molecule
cDNA	complementary DNA
CHS	Contact Hypersensitivity
COX	Cyclooxygenase
CPAF	N-methylcarbamyl platelet-activating Factor
CREB	cAMP response element-binding protein
CTL	Cytotoxic T Lymphocyte
DAMP	Damage-associated molecular pattern
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNFB	Dinitrofluorobenzene
ED50	Median Effective Dose
EGFP	Enhanced green fluorescent protein

EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
Fc	fragment crystallizable region of antibodies
GPC	Glycrophosphocholine
GPCR	G-protein coupled receptor
HBSS	Hank's Balanced Salt Solution
HRP	horseradish peroxidase
Ig	Immunoglobulin
IL	Interleukin
ILP	Isolated limb perfusion
IMDM	Iscove's Modified Dulbecco's Medium
i.p.	intraperitoneal
IP_3	Inositol Triphosphate
ITAM	immunoreceptor tyrosine-based activation motifs
K_D	Dissociation Constant
KO	Knock-out
LN	Lymph Node
MC	Mast cell
MCP/MCPT	Mast cell protease
MDSC	Myeloid-derived suppressor cell
MMP	Matrix Metalloprotease
mRNA	messenger ribonucleic acid

NAC	N-acetyl cysteine
Ox-GPC	Oxidized glcerophosphocholine
OVA	ovalbumin
PAF	Platelet-activating Factor
PAF-AH	Platelet-activating Factor Acetylhydrolase
PAFR	Platelet-activating Factor Receptor
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PIP_2	Phosphoinositol Diphosphate
PGE_2	Prostaglandin E2
PLA_2	Phospholipase A2
qRT PCR	Quantitative Real-Time PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCF	Stem cell factor
SNARE	soluble N- ethylmaleimide-sensitive factor attachment protein receptors
s.c./s.q.	subcutaneous
TCR	T cell receptor
TGFβ	Transforming growth factor β
Th	T helper cell
Treg	Regulatory T cell
UVB	Ultraviolet light B
UCA	Urocanic acid

Chapter 1: Introduction and Literature Review

I. PAF

As a result of tissue damage, infection, or exposure to immunogenic molecules, the body coordinates inflammatory responses by orchestrating various cell types through the release of soluble mediators [1]. These soluble mediators drive the balance between pro-inflammatory and anti-inflammatory mechanisms. Platelet-activating factor (PAF) is a soluble lipid mediator of both local acute inflammation as well as delayed systemic immunosuppression [2-8]. Still, the mechanism of PAFR-mediated immunosuppression remains to be fully elucidated.

A. PAF physiology

PAF (1-hexadecyl-2-acetyl-glycerophosphocholine, Figure 1) is among the most potent phospholipids known, inducing asthma-like responses in the low picomolar range [9], and resulting in anaphylaxis at higher doses [10]. PAF was first described when Henson proposed in 1971 that there was a leukocyte-derived soluble mediator that activated platelets [11]. It was not until 1972, however, when PAF was first identified as the mediator released by stimulated basophils that resulted in platelet aggregation [12]. This activity is due to multiple glycerophosphocholines (GPCs), with 1-hexadecyl-2-acetyl GPC being the most potent. Expression of its G-protein coupled receptor (GPCR), PAFR (*Ptafr*), has been demonstrated in many tissues and cell types, including: platelets, neutrophils, macrophages, dendritic cells, langerhans cells, keratinocytes, mast cells, neurons, myometrium, vascular endothelial cells and tracheal epithelial cells [13, 14]; and also many tissues such as: brain, kidney, skeletal muscle, smooth muscle, and alveoli in the lung [13].

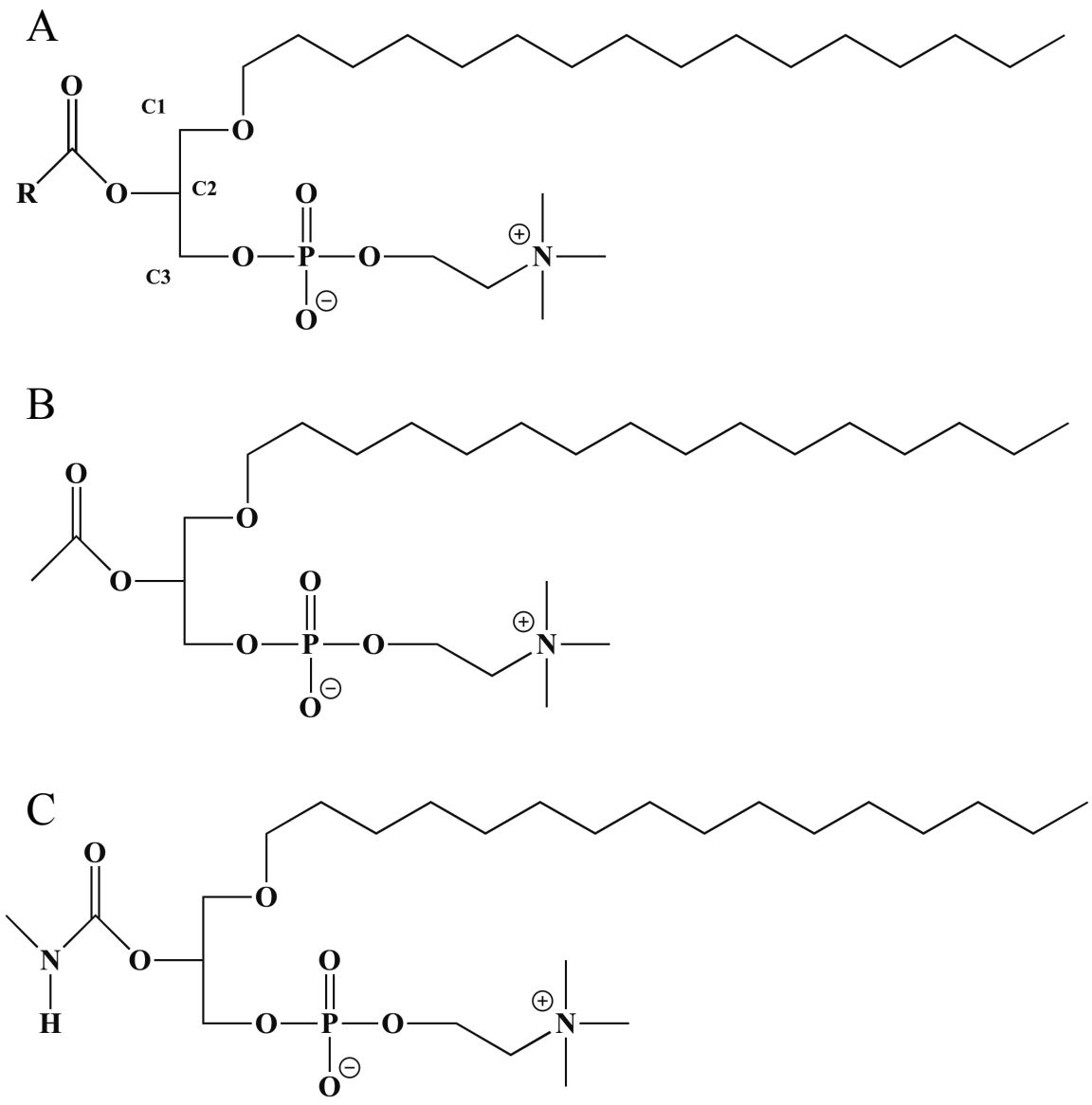


Figure 1. Structure of GPC, PAF and CPAF.
A. General structure of GPC. Glycerol position C1 (*sn-1*) consists of hexadecyl ether group, C2 (*sn-2*) is an acetyl linkage (typically arachidonate), C3 (*sn-3*) is a phosphocholine group. **B.** The structure of PAF. **C.** The structure of N-methylcarbamyl PAF.

PAF stimulates the activation of platelets, and leukocytes in response to traumatic, allergic, and infectious events [15-17]. In particular, PAF has been shown to promote platelet aggregation, induce smooth muscle contraction, initiate bronchoconstriction and tracheal mucus secretion, promote vasoconstriction, vasodilatation and vascular leakiness, promote neuron maturation and synapse formation at physiologic concentrations, and induce neuronal, astrocyte, and oligodendrocyte apoptosis at supraphysiologic concentrations [4-6, 10, 15]. Relevant to the immune system, PAF is known to induce macrophage, neutrophil and eosinophil chemoattraction,[18-20] and neutrophil extracellular trap release [21]. B lymphocyte cell lines also express functional PAFRs [22]. While, the expression of PAFR on T lymphocytes remains controversial [5], we have evidence to support the absence of PAFR expression in T cells.

PAF is released after mast cell, endothelial cell, platelet, neutrophil, basophil, and macrophage activation, as well as following reperfusion injury and ischemia [4-6, 10, 15, 23]. PAF is synthesized under tightly regulated enzymatic pathways (Figure 2), as well as unregulated mechanisms mediated by oxidative stressors (Figure 3). Under physiological conditions, phospholipase A_2 cleaves the fatty acid moiety on the second carbon in the glycerophosphocoline glycerol backbone to generate a lyso intermediate, which undergoes acetylation by PAF synthases to generate enzymatically synthesized PAF. Alternatively, PAFR agonists readily form non-enzymatically via the free-radical oxidation of GPCs to render oxidized-GPCs (ox-GPCs). This process is mediated by reactive oxygen species (ROS) generated by pro-oxidative stressors, such as ultraviolet light B (UVB) and cigarette smoke [24, 25].

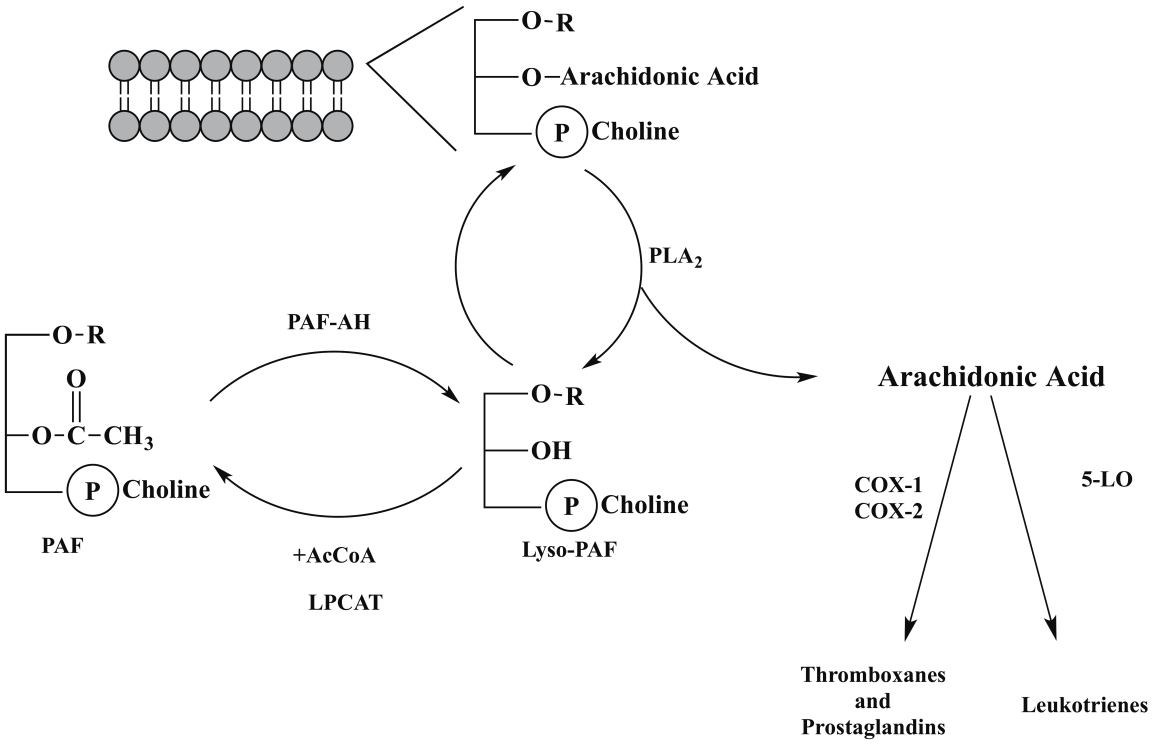


Figure 2. The enzymatic synthesis of PAF.
GPCs from lipid membranes are cleaved at the glycerol C2 (*sn-2*) position by PLA_2, liberating arachidonic acid and Lyso-PAF. Free arachidonic acid is then converted to leukotrienes by 5-lipooxygenase, or thromboxanes and prostaglandins by COX-1 or COX-2. Lyso-PAF is acetylated by LPCAT to form PAF. This acetylation reaction is reversible by the cleavage of the C2 acetyl group by PAF-AH forming lyso-PAF which is then re-acylated to form GPC or re-acetylated to form PAF.

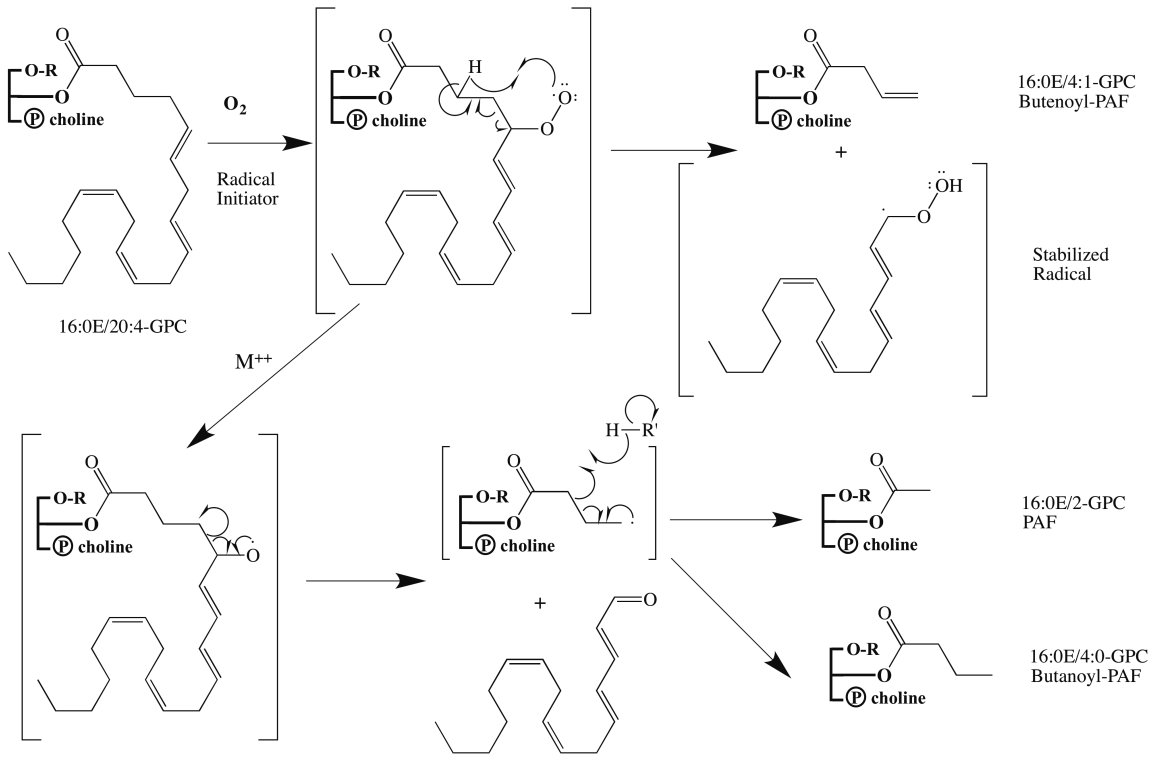


Figure 3. Proposed outcomes of PAFR-agonistic ox-GPC formation.
Basal state triplet oxygen ($^3O_2$) is activated to singlet oxygen ($^1O_2$) or superoxide radical ($O_2^{\bullet-}$) by pro-oxidative stressors. Superoxide radical can then be resolved to other ROS including hydrogen peroxide and hydroxide radical. These ROS oxidize unsaturated bonds in GPCs, predominantly arachidonate in the C2 glycerol position due to rich π-electron bonding systems. These intermediates can resolve in many ways including butenoyl-PAF and a stabilized radical (above), or by metal (M^{++}) catalysis resolve to give PAF, butanoyl-PAF, among many other possible mechanisms.

B. Structure and synthesis of PAF

PAF (Figure 1) is derived from GPCs, the major component of lipid membranes in cells and organelles. PAF is made up of a phosphocholine group on the *sn-3* position of a glycerol backbone, while an ether-linked alkyl and an acetyl group fill *sn-1* and *sn-2* positions, respectively. Under physiological conditions, phospholipase A_2 (PLA_2), under the control of Ca^{2+}, ERK and p38, cleaves the fatty acid moiety on the *sn-2* position of the GPC glycerol backbone to generate a lyso intermediate. This intermediate undergoes acetylation by PAF synthases, such as lysophosphatidylcholine acyltransferase to generate enzymatically synthesized PAF. Alternatively, PAFR agonists readily form non-enzymatically via the free-radical oxidation of GPCs, a process mediated by ROS generated by oxidative stressors, such as UVB and cigarette smoke. The mechanism of this reaction has been explored in some detail [24-26]. Generally, ROS react with and oxidize unsaturated groups in the fatty acid chain on the *sn-2* position of GPCs. The products that result from the resolution of this unstable intermediate can result in the cleavage of the *sn-2* fatty acid and a variety of PAF-like ox-GPCs (Figure 2). This reaction is favorable because fatty acids in this position, typically arachidonic acid, have π-electron rich bonding systems that are highly reactive with ROS. Once synthesized, however, PAF agonists can be broken down into their lyso intermediate by circulating PAF-acetyl hydrolases (PAF-AH, also known as lipoprotein-PLA_2), or by intracellular PAF-AH II via the hydrolysis of the *sn-2* ester bond [27]. Experimentally, it has been shown that the half-life of circulating PAF is less than 7 minutes in humans and 30 seconds in mice [28]. For this reason, N-methylcarbamyl-PAF (CPAF), a metabolically stable PAFR agonist, is used in research.

C. Structure and pharmacology of the PAFR

The effects of PAF in anaphylaxis and asthma have long been recognized, while the identification of the PAFR remained elusive until it was first cloned in 1991 [29]. The PAFR is highly specific to PAF and binds PAF with a K_D (binding affinity) of 0.61 nM [30]. ED50 values have been demonstrated to range in the low nM range [8]. Hydropathy analysis demonstrated the presence of seven hydrophobic transmembrane domains in the protein sequence of PAFR, consistent with the structure of a GPCR [29]. This evidence was consistent with observations that the effects of PAF are mediated by G-proteins [3]. The PAFR gene is regulated by two sets of promoters on the 5' non-coding region of the gene [5]. This gives rise to two different PAFR transcripts that are differentially regulated and are hypothesized to have different signaling pathways.

Early work on this field found that heterotrimeric G-proteins bind a ligand-bound PAFR resulting in the release of stored calcium, decrease in cyclic AMP, an increase in inositol triphosphate and release of arachidonic acid (Figure 4) [3]. Studies have shown that Gαi classically mediates a decrease in cAMP by inhibition of adenylate cyclase, following PAFR activation. Furthermore, it has been shown that PAFR mediates activation of: Erk by Gαq/11, Gαo and Gβγ, PLCβ by Gαq/11 and Gαo, and p38 by Gαq/11. Interestingly, PAF has been shown to mediate some effects via non-G-protein dependent pathways. In particular, Jak/STAT pathway activation has been demonstrated to be the result of Tyk activation by PAFR (Figure 4). These intracellular signaling pathways have been implicated in various cell-specific effects ranging from chemotaxis to survival and proliferation.

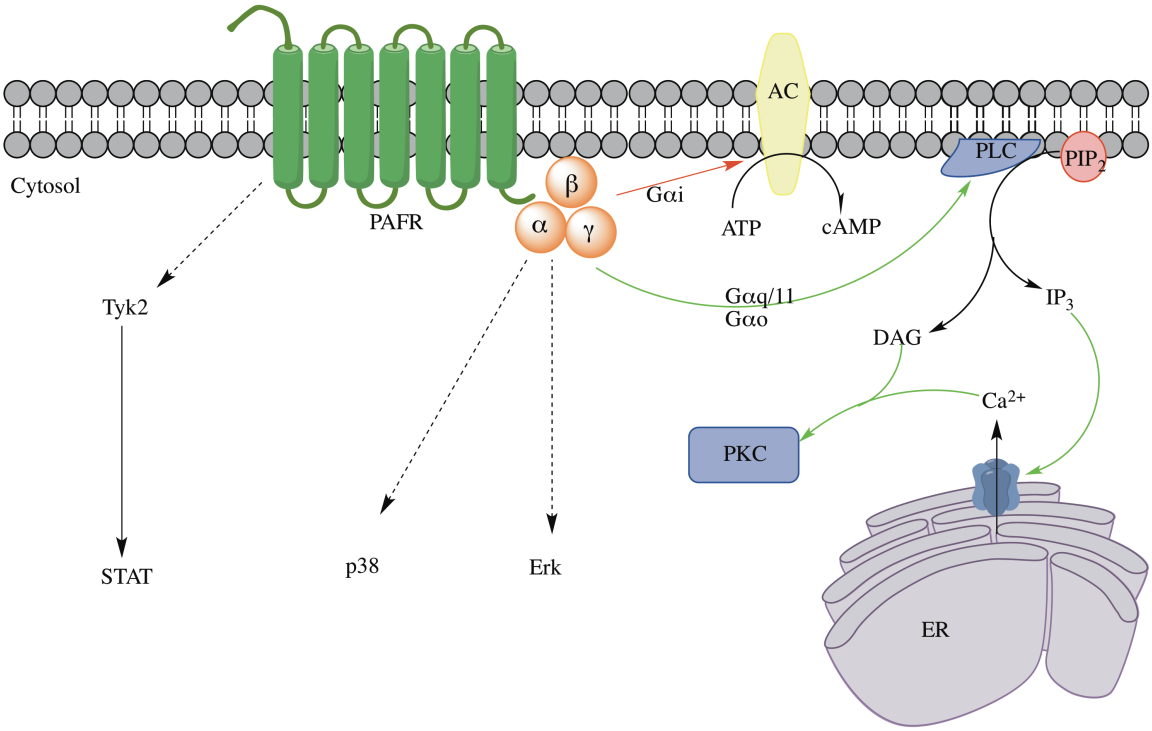


Figure 4. Intracellular signaling following PAFR activation.
Activation of PAFR stimulates G-protein and non-G-protein mechanisms. PAFR can activate Tyk2/STAT pathways via independent of G-proteins. Alternatively, PAFR can activate (green arrows) or repress (red arrows) secondary messengers via G-protein activation. PAFR activation can repress adenylyl cyclase (AC) via Gαi, and activate phospholipase C (PLC) via Gαq/11 and Gαo. Activation of PLC results in cleavage of PIP_2 to render free DAG and IP_3, which activate PKC by sequestering PKC to the cell membrane and inducing intracellular calcium release, respectively. Additionally, PAFR can activate p38 and Erk MAP kinase pathways via G-protein dependent mechanisms.

Additionally, several lines of evidence have implicated the role of epidermal growth factor receptor (EGFR) in mediating cell proliferation (Figure 5) [31-33].

Cloning the PAFR made the generation of PAFR overexpressing and PAFR deficient (KO) mice possible, which was accomplished by Satoshi Ishii in 1997 and 1998, respectively [34, 35]. The PAFR overexpressing mouse was generated by inserting a transgenic copy of the PAFR cDNA under the control of a CMV enhancer and β-actin promoter. Interestingly, these mice were found to demonstrate increased endotoxin sensitivity, increased bronchial reactivity, and greater spontaneous melanocytic tumorigenesis [34]. The PAFR KO mouse was generated by disrupting the PAFR gene with a PGK-neo cassette insertion into the open-reading frame. This mouse was shown to develop and reproduce normally, and maintains sensitivity to endotoxin, but has decreased sensitivity to anaphylaxis by intravenous OVA administration in OVA-sensitized mice [35]. Other studies have shown that this mouse may be predisposed to obesity with age and may be protected from oophorectomy–related osteoporosis. More recently, however, this mouse has been shown to be resistant to UVB-induced systemic immunosuppression [36].

D. PAF: pro-inflammatory and anti-inflammatory homeostasis

One of the essential characteristics of all living organisms is the need to maintain homeostasis. For complex organisms, this includes mounting robust immune responses to fight off infection or repair tissues, but also having the ability to attenuate this response when the injury or infection resolves. The inability to mount an appropriate immune response can leave the organism vulnerable to lethal disseminated infection. This is highlighted in patients with severe immunodeficiency disorders.

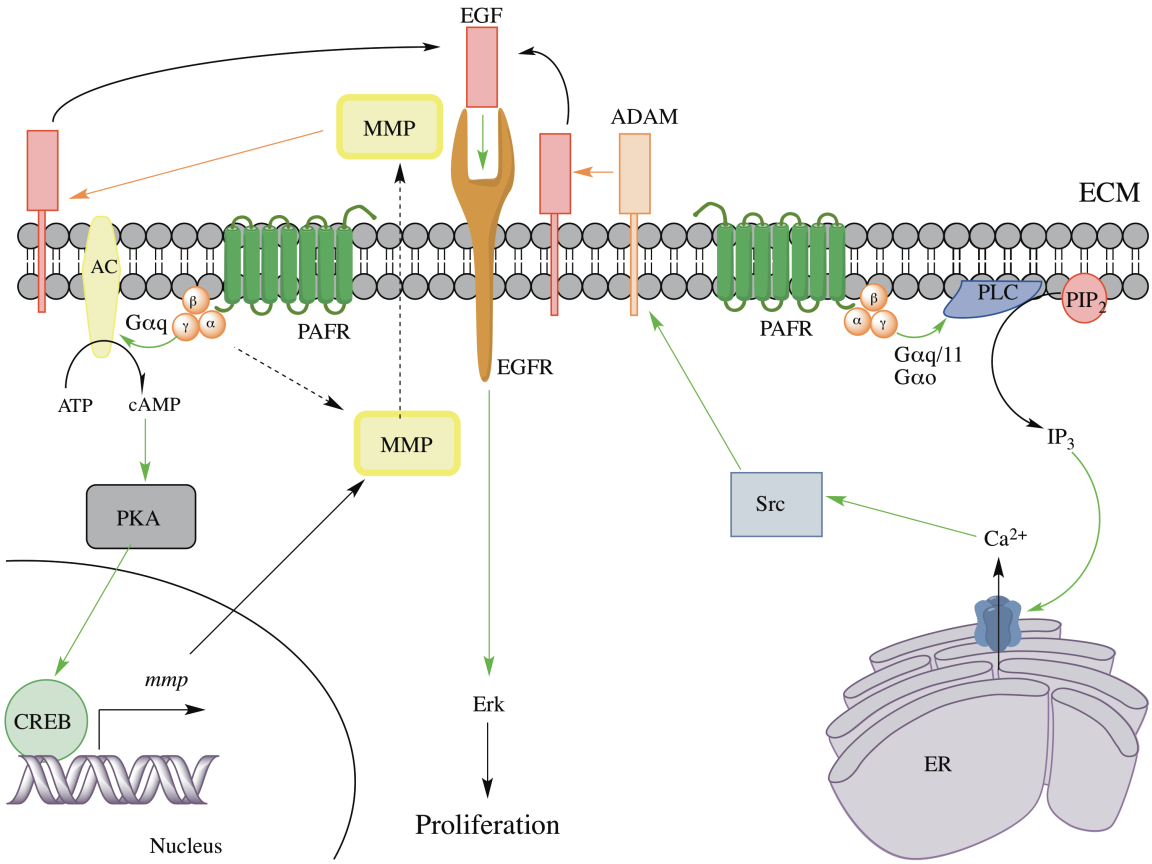


Figure 5. Extracellular signaling following PAFR activation.
Activation of PAFR stimulates signal transduction via EGFR-mediated mechanisms. PAFR can activate (green arrows) PLC and AC pathways through Gαq activation. PLC activation leads to Ca^{2+} release which activates Src mediated activation of a disintegrin and metalloproteinase (ADAM). Activation of AC increases cAMP concentrations that activate PKA, which can induce the upregulation of metalloproteases (MMPs) via the phosphorylation of transcription factor cAMP response element-binding protein (CREB). Released MMPs and activated ADAMs can cleave (orange arrows) surface bound epidermal growth factor (EGF), which activate membrane-bound EGFR. EGFR induces cell proliferation and cell survival via the phosphorylation of Erk.

On the other hand, the inability to attenuate an inflammatory response predisposes the organism to chronic inflammatory disease, carcinogenesis or autoimmune disease. For this reason, maintaining inflammatory balance is necessary. While, the importance of PAF in the immune system and immune responses is irrefutable, its seemingly bivalent role is still a focus of investigation. As previously discussed, PAF is known to mediate robust pro-inflammatory mechanisms both locally, such as urticaria and bronchoconstriction, as well as systemic anaphylaxis. With the development of a PAFR KO mouse, however, the role of PAF as an anti-inflammatory mediator has come to light.

II. The immune system

Mammals have complex immune systems consisting of both pro-inflammatory as well as anti-inflammatory mechanisms, topics that require whole textbooks to cover in detail [1]. Inflammatory mechanisms are largely mediated by inflammatory effector cells, such as neutrophils, macrophages, cytotoxic T lymphocytes (CTLs) and many T helper subsets, through the release of inflammatory mediators, such as TNFα, interleukin-4 (IL-4), and IFN-γ. On the other hand, anti-inflammatory mechanisms are mediated largely by regulatory T cells (Tregs), a subset of CD4+ T helper cells, through the release of anti-inflammatory mediators, such as TGFβ, IL-10, and prostaglandin E_2 (PGE_2). These responses typically involve two main branches of the immune system, the innate and the adaptive immune system. Cytokines and chemokines orchestrate the mechanisms between these two branches.

A. Cytokines and chemokines

In short, cytokines are proteins that signal between cells in the immune system and the rest of the body [37]. Most cells in the body can release cytokines to signal the

promotion or attenuation of inflammatory responses. Cytokines can activate and recruit cells, affect vasculature to promote or reduce swelling, and induce fever. As mentioned above, inflammatory cytokines include TNFα, IL-4, and IFN-γ, whereas anti-inflammatory cytokines include TGFβ and IL-10. Moreover, cytokines can also be divided into Type 1, and Type 2 cytokines. Type 1 cytokines, such as IL-2 and IFNγ, promote cell-mediated immune responses. Type 2 cytokines, such as IL-10 and IL-4, promote humoral, or antibody-mediated immunity. Chemokines are chemotactic cytokines that induce cell motility of specific chemokine receptor-expressing cells toward a concentration gradient of the chemokine ligand. An example of this is the trafficking of CCR7+ dendritic cells to CCL19-releasing LNs following dendritic cell activation [38].

B. The innate immune system

The most basic function of the immune system is the ability to distinguish between self and foreign organisms [39, 40]. While the adaptive immune system is responsible for identifying foreign proteins in the context of self, this is a relatively slow process taking up to a week to mount an immune response. The innate immune system, however, mediates responses within hours and is a critical first line of defense. By 24 hours, the innate cells infiltrate the site of infection or damage, and begin clearance of damaged cells, noxious agents, and pathogens. During this process, some cells called antigen presenting cells (APCs) take up foreign proteins, process them, and present them to cells of the adaptive immune system over the course of several days to activate the adaptive immune response.

The innate system is classically composed of dendritic cells, macrophages, neutrophils, natural killer cells, eosinophils, basophils and mast cells (MCs). Of these

cells, dendritic cells and macrophages are the main APCs that present antigen to the adaptive immune system. Neutrophils, macrophages and natural killer cells are mostly involved in foreign body, apoptotic body and pathogen clearance. Eosinophils, basophils and MCs are responsible for promoting and sustaining pro-inflammatory mechanisms.

Innate cells are primarily activated and recruited by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [41]. PAMPs are molecules, usually components of pathogen cellular structure, which are unique to a particular group of pathogens and thus help in differentiating host from pathogen. PAMPs have several specific receptors that they activate, which are called toll-like receptors. A classical example is lipopolysaccharide that is a component of gram-negative bacteria cell walls, binds toll-like receptor 4 [42]. DAMPs are typically host intracellular components that upon release, as a result of necrosis, cell damage or exocytosis, send a danger signal to the innate immune system signifying that pathogens or damaged cells need to be cleared. DAMPs activate several receptors that are a current topic of investigation. An example of a DAMP is HSP90, an intracellular protein chaperone that is released upon cell stress and activates CD91 on innate cells [43].

Recently, the identification of an innate cell population with immunosuppressive potential has been revealed [44, 45]. Originally discovered in the 1960s in tumor models, myeloid-derived suppressor cells (MDSCs) appear to be a heterogenous population of cells that contribute to the immunosuppressive branch of the innate system [46]. They are primarily known for promoting tumorogenesis and metastasis [47, 48], but have been shown to have effects in other systems [45]. While lacking markers for T cells, B cells, NK cells and macrophages, MDSCs are typically characterized as antigen Gr1+ CD11b+

in mice and CD14–CD11b+ in humans [44]. MDSCs have been shown to interact closely with other innate cells, such as MCs, but also with the adaptive immune system [49-51].

C. The adaptive immune system

While the innate immune system is limited to recognizing PAMPs and DAMPs, it is estimated that the adaptive immune system is able to recognize over $2.5x10^7$ different antigens, or specific peptide sequences [52]. The process of identifying pathogen antigens and mounting an immune response takes time, but results in a specific and efficient response. The adaptive immune system consists of two main cell types, B cells, which produce antibodies, and T cells, which mediate and assist cell mediated immunity. Over the course of several days following infection, APCs will take up proteins and after receiving an inflammatory signal, will migrate to draining LNs where B and T cells await activation.

B [53] and T [54] cells are activated when their receptors (BCR and TCR, respectively) bind a specific antigen. In the LN, B and T cells express unique receptors that are specific to a particular antigen. Following presentation of the antigen on major histocompatibility complex (MHC) molecules by APCs, T cells expressing a TCR specific to the antigen being presented will be activated and rapidly proliferate, resulting in clonal expansion of antigen-specific T cells. Simultaneously, antigen-specific B cells will recognize soluble antigen on their BCR, receive a primary activation signal and internalize this complex. The antigen taken up is then presented to T cells. A cognate interaction occurs when antigen-specific mature T cells meet a matching activated B cell by virtue of the presented surface antigen. This interaction results in the secondary activation signal to B cells to initiate the production and release of soluble antibodies.

The antibodies can then neutralize pathogens by opsonization, decrease motility, or cell lysis by the activation of the complement system. Following this secondary activation, B cells can also be instructed to produce different isotypes of antibodies, each which have well defined functions. IL-4 for example, induces B cells to undergo class switching to IgE.

As the process of B cell activation and antibody production ensues, two main types of T cells also mediate the immune response: CD8+ CTLs [55] and CD4+ T helper (Th) [56] cells. Once activated, CTLs are responsible for direct cell mediated immunity. They travel to the site of insult and survey cells for the presence of the particular foreign antigen to which they were activated. If they find a cell that presents this peptide in the context of MHC, they will release cytotoxic granules that will induce apoptosis. In parallel, Th cells are mostly responsible for supporting various branches of the immune response. To tailor to different inflammatory scenarios, various subsets of Th cells develop that secrete different cytokines depending on the cytokine microenvironment. Historically, Th subsets were classified as Th1, Th2 and Tregs. Type 1 cytokine-producing Th1 cells, promote Type 1 immune responses or cell mediated immunity. They promote the expansion and activation of CTLs as well as recruitment of macrophages and neutrophils. Type 2 cytokine-producing Th2 cells, promote Type 2 or humoral immune responses. They induce the activation of B cells to produce soluble antibodies, and induce mast cell and eosinophil recruitment. As mentioned earlier, another subset of Th cells are Tregs, which mediate anti-inflammatory responses by the secretion of IL-10 and TGFβ. They inhibit T cell proliferation and promote wound healing. Since the definition of Th1, Th2 and Treg cells, many other subsets of Th cells have been identified and named

according to the interleukin that they express, such as Th9 and Th17, or their role, such as T follicular helper cells, Tfh.

After the necrotic tissue, noxious stimulus, or pathogen is cleared a normal immune response is attenuated [57, 58]. During this process, many immune cells succumb to apoptosis due to the regression of prosurvival and proliferative signals and cytokines. Apoptotic neutrophils, necrotic cells and remaining pathogens are cleared mostly by tissue resident macrophages. Over 90% of the antigen-specific T cells and B cells also perish by apoptosis. Some of the remaining lymphocytes continue to circulate for some time, while others receive signals to become endowed with longevity as memory T and B cells. These memory cells serve to quick start immune responses should the host encounter this antigen again, reducing the time to ramp up adaptive immunity to a couple of days. Finally, some B cells will maintain some antibody production that will circulate in the blood for some time. IgE antibodies produced will bind specific IgE-receptors, FcεR, on basophils and MCs.

III. The mast cell

Mast cells were discovered by Paul Ehrlich in the late 1870s. He described "mastzellen" as granulated aniline staining cells that resided in perivascular connective tissues [59-61]. Since then, MCs have been found to be long-lived hematopoietic cells that serve as sentinels in interfaces with the environment and circulatory system to initiate allergic responses upon recognition of pathogenic stimuli. In particular, MCs are a necessary part of the immune system to clear toxins, and fight off parasitic, e.g. helminth, and bacterial infections [62]. Most notably, however, MCs are also responsible for seasonal allergies in millions of people worldwide [63, 64]. 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, in addition to the production of eicosanoids, such as prostaglandins and leukotrienes. While their pro-inflammatory role is well documented, lately their role as anti-inflammatory mediators is a current topic of investigation.

A. Mast cell development and trafficking

MCs originate in the bone marrow from a common hematopoietic stem cell progenitor [65]. Common myeloid progenitor cells will differentiate from this stem cell pool and finally give rise to MC progenitors. These stem cell and MC progenitor populations expand in response to the activation of the stem cell factor (SCF) receptor, cKit. These MC progenitors exit the bone marrow and circulate the blood until they extravasate into tissues where they will mature into MCs that are characteristic of that tissue, while still maintaining cKit expression. Integrins and chemokine signaling largely regulate this migration. An example of this is the recruitment of MC progenitors to the gut, where CXCR2 was found to be necessary [66]. Once in the tissue, MCs have been estimated to have a life span of over 9 months [67]. This process of tissue maturation is advantageous for transplant models, where in vitro-derived MCs can be transplanted to reconstitute tissues with mature populations of tissue resident MCs. Mast cell reconstitution in MC-deficient mice has been well characterized and utilized in the literature [68-71]. Historically, it was thought that tissue-resident MCs remained in their tissues for the remainder of their life span. Recent evidence, however, suggests that MCs can migrate to nearby tissues after activation [72-74]. Their role after secondary

migration is a topic of current investigation and MC reconstitution models have proven to be useful.

There are different MC types whose characteristics depend on the tissue where they mature. Typically, MCs are classified depending on the contents of their granules. In mice, there are connective tissue MCs and mucosal MCs [75]. Connective tissue MCs, which include serosal MCs and skin MCs, typically have high granule density that contain heparin, histamine and MC proteases such as mMCP-4 and -5 (chymases), and mMCP-6 and -7 (tryptases). Mucosal MCs have low granular density that contain low levels of histamine and heparin, but high concentrations of mMCP-1 and -2 (chymases). Similarly, in humans, MCs are also divided into two groups characterized by their granular content: Tryptase+ MCs and Tryptase+ Chymase+ MCs. Interestingly, there has been some evidence pointing out the plasticity of MCs, where MC subtypes could become another MC type. An example of this was shown where heparin+ peritoneal MCs lost their heparin expression in vitro, but regained this function after transplantation [76].

B. MC signaling pathways

MCs function as signaling relays that translate incoming stimuli and orchestrate a myriad of responses through the release of secondary mediators. MCs have been shown to express an exhaustive list of receptors that include: Fc receptors, toll-like receptors, GPCRs (e.g. PAFR, histamine receptors, prostaglandin receptors), transient receptor potential cation channels, interleukin receptors, chemokine receptors, and intracellular receptors. Classically, mast cells are known to bind the Fc (fragment crystallizable) domain of circulating IgE antibodies on surface FcεRs. These bound IgE antibodies serve as sensors to detect antigens of possible pathogens. This makes Fc receptors versatile to

mediate responses to a large number of different antigens. When these surface bound IgE antibodies on MCs are cross-linked by an agonistic multivalent antigen, they cluster FcεRs. While, FcεRs lack intrinsic tyrosine kinase activity, secondary kinases are crucial to transphosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) on coupled neighboring receptors [77]. Identified secondary kinases Hck, Fyn, Syk and Lyn are Src family kinases. These Src kinases mediate the phosphorylation of ITAMs on FcεRs as well as the activation and deactivation of tertiary signal transduction pathways. This activation finally culminates in calcium mobilization, activation of gene transcription and translation, the synthesis and release of protein and lipid mediators, and MC degranulation. The rapid release of granule-stored mediators is the orchestration of calcium release and activation of actin, microtubule and SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) protein mechanisms [77-79].

C. MCs in physiology

Many of the granule contents released upon FcεR activation, induce potent pro-inflammatory effects. As mentioned before, granule contents include amines (e.g. histamine, polyamines), proteoglycans (e.g. heparin), proteases (e.g. tryptases, chymase, cathepsin, granzyme B, carboxypeptidases), lysosomal enzymes, and cytokines (e.g. TNF, FGF, IL-4 and SCF) [80]. These released mediators induce local leukocyte recruitment and infiltration, vasodilatation, and edema. Systemic activation of MCs, however, can result in anaphylactic responses. Proper MC activation can mediate successful countermeasures against infection; however, improper MC activation to innocuous stimuli like pollen, can result in allergic hypersensitivity reactions, such as

seasonal allergies. Interestingly, MCs have been shown to have more direct effects on the adaptive immune system. For instance, MC granules have been shown to travel through the lymphatic system to deliver granule contents to draining LNs [81]. In addition to mediator release, MC migration to draining LNs has been found to be important to induce immune responses [72]. MCs can phagocytose, express MHC-II and present antigen to influence T cell activity [82-85]. This evidence suggests that MCs can have non-canonical effects on the immune system. In fact, MCs have also been shown to attenuate immune responses [70].

D. MCs in immunosuppression

The suppressive effects of MCs on immune responses have some precedence [86, 87]. MC IL-10 release has been shown to limit pathology from contact dermatitis and chronic UVB irradiation [88]. MCs have been shown to contribute to transplant tolerance [89]. Additionally, experiments have demonstrated that MCs will induce Treg differentiation via TGFβ when cocultured with naïve T cells [90], and augment the suppressor activity of MDSCs [50, 91]. Still, it seems that MCs can fine tune immune responses even by transiently attenuating Treg function through histamine receptor H1 activation [92], but inducing Treg recruitment by H4 receptor activation [93]. Interestingly, MCs have been shown to attenuate anti-tumor immunity to promote skin tumorognesis [94, 95] and mobilize Tregs and MDSCs in the tumor microenvironment [96].

IV. Melanoma

In the United States, one in five people will develop a form of skin cancer during their lifetime [97]. Less than 1% will develop melanoma skin cancer; however,

melanoma claims more than 70% of skin cancer-related deaths [98]. Surgical excision cures virtually all melanoma in situ. Still, a survival rate of less than 15% for metastatic melanoma reflects deficiencies in current treatments [98]. Recent clinical reports show, however, that immunotherapies hold promise for treating metastatic melanoma [98-103]. Yet, these therapies depend on a competent immune system.

A. Pathophysiology of melanoma

There are two main predispositions for melanoma: sunlight exposure and genetic predisposition [104]. Sunlight exposure poses a threat due to the mutagenic effects on sunlight, particularly in its ability to form pyrimidine dimers in DNA [104]. These insults can propagate further mutagenesis in DNA that can eventually lead to cancer, especially if the genes involved are tumor suppressors or oncogenes [104]. It is well recognized that light skinned individuals are at greater risk for developing skin cancers, including melanoma. Moreover, sun exposed skin, such as upper back skin in men, and back and leg skin in women, are at greater risk for developing melanoma [104]. Still, the correlation between sunlight and carcinogenesis is not simple, for people occasionally develop melanoma in areas not associated with heavy sunlight exposure. Recently, however, it was found that heavy sunburns early in life might be of greater significance for melanoma formation [104].

Studies have estimated that greater than 10% of melanoma cases are familial [104]. In the case of hereditary and spontaneous melanomas two main signaling pathways are usually involved, either mutations diminishing tumor suppressors or mutations enhancing oncogenes. Approximately 40% of familial melanoma cases are found to have mutations in the *CDKN2A* gene, which encodes a complex locus of three different tumor

suppressors [104]. These tumor suppressors, p15/INK4b, p16/INK4a, and p14/ARF, ultimately activate other tumor suppressors, such as p53, which downregulates the activity of MDM2, a strong oncogene. The other group of familial melanoma cases consists of mutations promoting oncogenes. In particular, a large majority of these tumors have activating mutations in the RAS signaling pathways. For instance, 70% of melanoma tumors contain activating mutations to the oncogene BRAF, one of the targets of RAS [104]. As such, BRAF inhibitors are currently being developed and some currently available are first line of treatment.

B. Prognosis and current treatments

While surgery is the first line of treatment for melanoma in situ with great prognosis, therapeutic options for metastatic melanoma are limited. Metastatic melanoma is tolerant to chemotherapy and radiation therapy [105, 106]. Metastatic melanoma poses a grave prognosis due to a higher mortality rate, albeit less prevalent, than non-melanoma skin cancer. In 2010, the American Cancer Society estimated a disturbing 68,000 new melanoma diagnoses, whereas 8,700 deaths were calculated (www.cancer.org/statistics). Sadly, while incidence rates have declined slightly, medical advances prove inadequate to taper the raising mortality rate of melanoma [105-108]. As such, novel therapeutic approaches merit attention.

Currently, immunotherapies show promising results in the clinic, which include the use of cytokines, such as interferon, IL-2, IL-15, IL-21 and CTLA-4 (Cytotoxic T-Lymphocyte Antigen-4) inhibitors [109-112]. Adoptive transfer of melanoma specific T cells has also been used [100], as well as immune sensitizers, such as imiquimod. Thus, investigation of the interplay between melanoma and the immune system is relevant.

Recently, the use of oncogene-targeted therapy has been a growing field. BRAF inhibitors, such as vemurafenib, and tyrosine kinase inhibitors, such as imatinib, can also be used in tandem with immunotherapies.

C. Chemotherapy as a therapy and oxidative stressor

The mechanisms by which melanoma is resistant to chemotherapy is a topic of ongoing study. Still, chemotherapy has a place as a last line of recourse. While systemic administration of current chemotherapeutics, such as etoposide or dacarbazine, may be useful to shrink tumors for surgical resection, they typically are not curative alone. The systemic doses needed to overcome resistance would be extremely toxic to patients. Fortunately for some patients with metastatic disease contained to a single limb, isolated limb perfusion (ILP) chemotherapy can be used [99, 113, 114]. By cannulating the artery and veins supplying blood to a limb, an external pump can be used to deliver high dose chemotherapy to the isolated limb. ILP has been used with significant curative success in these patients. This method is not without drawbacks, however. Due to their nature, chemotherapeutics are known to be cytotoxic, immunosuppressive and strong oxidative stressors. The ROS generated following ILP could diffuse systemically after treatment. While chemotherapy could be immunosuppressive via the cytotoxicity of proliferative hematological stem cells, I hypothesize that ROS generated by chemotherapy could also lead to systemic immunosuppression following a PAFR-dependent mechanism. Such an immunosuppressive mechanism from ROS is a current topic of investigation in immunosuppression mediated by ultraviolet light.

V. Ultraviolet light mediated systemic immunosuppression

The therapeutic properties of sunlight have long been recognized and have been used in the clinic. In particular, ultraviolet light B (UVB) is commonly used in the clinic. UVB is a component of sunlight, and is characterized as light in the wavelengths between 315-280 nm, whereas the visible spectrum is made up of wavelengths between 760 – 400 nm [115]. In the clinic, UVB has been used to treat vitiligo and psoriasis, both autoimmune diseases in which the immune system is self-reactive to melanocytes and keratinocytes, respectively [116, 117]. These phototherapies are particularly effective largely due to their immunosuppressive effects, which have been an ongoing topic of research. To date, several pathways have been identified that mediate the immunosuppressive effect of UVB. DNA, Cis-urocanic acid (cis-UCA), and PAF are among these pathways [118, 119].

A. Contact hypersensitivity models

Several models have been proposed to study the immunosuppressive effects of UV light. The most common in vivo model for the field is a contact hypersensitivity (CHS) model. CHS is a Type IV hypersensitivity, also known as delayed- or cell-mediated hypersensitivity, which is a type of reaction that is classically seen with poison ivy reactions. In contrast to type I hypersensitivities, such as seasonal allergies that are antibody-mediated, CHS reactions are mediated largely cell-mediated both by CD8+ CTLs and CD4+ Th1 cells. A typical CHS reaction in mice has two phases, a sensitization phase and a challenge phase (Figure 6).

During the sensitization phase, the antigen is introduced either topically or subcutaneously. Over the course of 5-9 days, APCs in the skin, Langerhans cells in the

epidermis and dendritic cells in the dermis, will uptake the antigen, migrate to draining LNs, and process and present the antigen on surface MHC molecules. In the LN, APCs present the antigen to both CD8+ and CD4+ T cells, where antigen specific cells will be activated and mature into CTL and various T helper cells, respectively. Most of the CD4+ T cells will polarize and differentiate into Th1 cells that promote cell-mediated immunity by producing Type 1 cytokines, such as IL-2 and IFNγ. IL-2 is particularly important for the expansion of the activated CTL population to promote inflammation and clearance of the noxious antigen. Some CD4+ T cells will also differentiate into Tregs, whose role it is to keep the inflammatory response in check by attenuating CTL activity by the release of TGFβ and IL-10. Toward the end of the sensitization phase, the antigen will be cleared and some of the activated T cells will develop into memory cells.

After the sensitization phase, the mice are then challenged with the same antigen, typically on a site different than the site of sensitization. During the course of 24 hours, APCs will present the antigen in draining LNs and reactivate memory T cells. This will lead to a quicker and more robust expansion of CTL populations than in the sensitization phase. These cells will then infiltrate the site of challenge and mediate a large immune response that promotes inflammation of the tissue, which can be quantitated. This model is useful to quantitate the effect of various treatments on cell-mediated immune responses. In fact, it is well documented that UV irradiation on the dorsal skin of mice before the sensitization phase significantly attenuates the subsequent antigen challenge [119]. This decrease in swelling is a measure of the immunosuppressive effects of UV light.

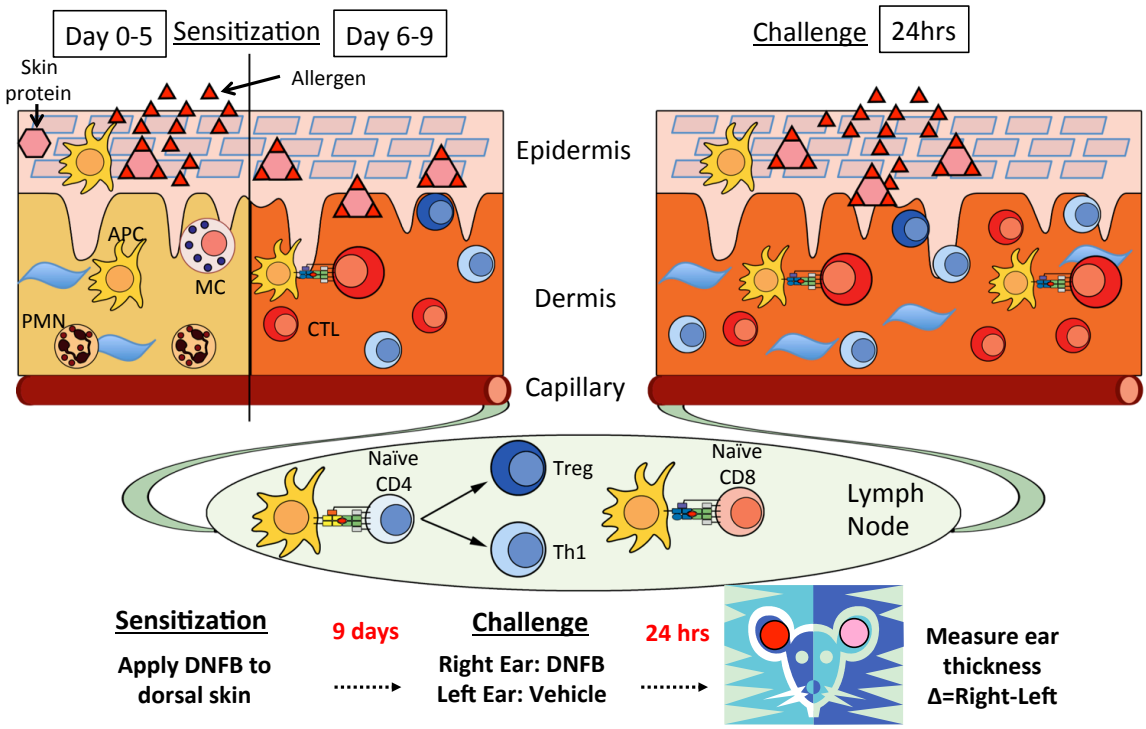


Figure 6. Mechanism of contact hypersensitivity reactions.
During the first exposure to an allergen, the immune system will become sensitized to the particular antigen in a process called sensitization. Hapten allergens, like DNFB, will bind proteins in the skin which are then taken up by antigen presenting cells (APCs) in the skin. The allergen will also induce activation of keratinocytes, MCs and fibroblasts in the skin, leading to the release of pro-inflammatory mediators. The release of these pro-inflammatory signals will induce the recruitment of PMNs and macrophages, as well as the migration of dendritic cells to LNs. Over the course of the next five days, APCs will present the allergen antigen to CD4+ and CD8+ T cells leading to their activation and expansion. Activated CD4+ T cells will differentiate into various T helper subsets, including Th1 cells which by the release of IFNγ and TNFα promote CD8+CTL cell-mediated inflammatory mechanisms, and Tregs which suppress the inflammatory response. These activated T cells will then migrate to the original site of sensitization over the course of days 6-9, and mediate inflammatory mechanisms by the release of IFNγ and TNFα. For CHS experiments, a second site is exposed to the allergen to induce a challenge response nine days after sensitization. To do so in mice, ears are measured, one ear is treated with the allergen DNFB, and the other is treated with vehicle. After 24 hours, ears are measured again. Over the course of these 24 hours, the adaptive immune system will mount a robust inflammatory response in the allergen treated ear via the infliltration of CTLs and Th1 cells.

B. DNA and Cis-UCA

The carcinogenic properties of UV light have long been recognized, due in part to the resulting pyrimidine dimer formation and subsequent mutagenesis in DNA. Work by Kripke, and collaborators, demonstrates that DNA can also act as a photoreceptor to initiate the immunosuppressive effects of UV light [120]. Using a CHS model, they found that if DNA repair pathways were activated after UV irradiation, the DNA damage was resolved along with the immunosuppressive effects of UV. Another photoreceptor that has been found is trans-urocanic acid, which is a deamination product of histamine found abundantly in the stratum corneum [118, 119, 121]. Upon UV irradiation, trans-urocanic acid isomerizes into cis-urocanic acid, which has been shown to mediate systemic immunosuppression in mice [122]. In fact, tape stripping to remove the stratum corneum in mice has been shown to inhibit the immunosuppressive effects of UV [123], a process that can be rescued by injection of cis-urocanic acid [122]. The ability of UV to isomerize urocanic acid and induce pyrimidine dimer formation is largely due to its properties as ionizing radiation. As ionizing radiation, however, UV can also induce ROS formation.

C. Free radicals, lipid oxidation and PAF

Due to its high-energy electromagnetic properties, UVB light has been shown to excite oxygen and water to form free radicals. For example, ground state triplet oxygen ($^3O_2$) can be activated to singlet oxygen ($^1O_2$) or superoxide anion ($O_2\bullet^-$). Superoxide can then be reduced to hydrogen peroxide (H_2O_2) spontaneously or by superoxide dismutase. Hydrogen peroxide can then be reduced further to form hydroxyl radical ($OH\bullet^-$) by heavy metals in solution (e.g. Fe^{2+}) via the Fenton reaction. These ROS while transient are

highly reactive and propagate radical formation in solution. In the skin, ROS can react with any number of targets including carbohydrates, nucleic acids, proteins, and lipids. Some of the most abundant targets are phospholipids that make up outer and organelle membranes in cells. As explained above, these ROS can induce the formation of PAF-like species by oxidizing GPCs in cell membranes.

PAF, as well as ox-GPCs, have been identified as primary mediators of the immunosuppressive effects of UVB. First, Ullrich and colleagues demonstrated that exogenous PAF is sufficient to activate systemic immunosuppression [16]. Later on, our lab demonstrated that UVB generates PAF-like phospholipids [26] and that these phospholipids induce systemic immunosuppression. Work in this field has shown that systemic administration of antioxidants can attenuate the immunosuppressive effect of UVB on CHS as well as the generation of ox-GPCs in vitro. Furthermore, structural identification of many ox-GPCs induced by UVB irradiation has been made possible by mass spectrometry analysis. The ability of UVB to act as an oxidative stressor to induce the formation of PAF-like species has also been demonstrated in other oxidative stressors. Such other oxidative stressors that induce PAFR-dependent systemic immunosuppression are cigarette smoke, jet fuel and photodynamic therapy [118, 119]. Mechanistic studies for PAFR-mediated immunosuppression are ongoing, but several mediators of PAFR-mediated immunosuppression have been identified.

D. Mediators involved in PAFR-mediated immunosuppression

In recent years, Tregs have been shown to mediate immunosuppression. Growing evidence suggests that Tregs are a necessary component for UVB-induced immune suppression [118, 119]. Recent studies by our lab have shown that Tregs play a crucial

part in the PAFR-dependent pathway of immunosuppression [24, 25]. In fact, this effect can be attenuated by the treatment with Treg-depleting antibodies. Tregs have been shown to increase in populations following CPAF and UV treatment. Furthermore, UV-generated Tregs can be adoptively transferred to a naïve organism to establish tolerance [124]. IL-10, one of the primary mediators of immunosuppression released by Tregs, has also been implicated in this pathway. IL-10 neutralizing antibodies significantly reduce the amount of PAFR-dependent immune suppression. Another cell type that has been implicated in this pathway is the mast cell [124-127].

Upon activation, mast cells release a number of immune modulators by de novo synthesis of mediators, or degranulation of pre-made vesicles. In addition to expressing the PAFR, MCs have been shown to release histamine and prostaglandins, as well as an upregulation in a number of chemokines and chemokine receptors upon activation [65, 72, 128, 129]. Interestingly, while wild type mice demonstrate sensitivity to PAF-induced systemic immune suppression, this sensitivity is ablated in mast cell deficient mice. Reconstitution of dermal WT MCs, however, restores the immune suppressive response to PAF. Additionally, work by Ullrich and collaborators demonstrate that MC migration to draining lymph nodes (LN), following systemic PAF treatment, is necessary for PAF-induced immunosuppression [73, 74]. Furthermore, this MC migration and subsequent immunosuppression was attenuated using a specific inhibitor to the CXCL12 chemokine receptor CXCR4, AMD3100 [73, 74]. This evidence suggests that MC CXCR4 is necessary to mediate MC migration in PAF-induced immunosuppression. This migration to lymph nodes could allow for direct MC and T cell interactions for the possible

generation and activation of Tregs. The mechanisms by which CXCR4 and CXCL12 are orchestrated are still a topic of investigation.

One proposed mechanism of action involves the role of prostaglandins, in particular PGE_2. As mentioned previously, prostaglandins are synthesized from arachadonic acid released from GPCs by the cleavage of PLA_2. This free arachidonic acid is then converted to PGG_2, by the prostaglandin synthase activity of COX, and reduced to PGH_2 by the peroxidase activity of COX. PGH_2 can then be converted to various other prostaglandins by the activity of other prostaglandin synthases, such as PGE_2 by prostaglandin E synthase. There are classically two isoforms of COX, the constitutively active COX-1 and the inducible COX-2. One of the main differences between COX-1 and COX-2 is that COX-2 has a greater binding affinity for PGES and generally increases the production of PGE_2, PGD_2, and $PGF_2\alpha$. A significant body of work has demonstrated that UVB and PAF can induce the expression of COX-2, and that it is necessary for the subsequent immunosuppression [24, 25, 130]. For instance, there is evidence that systemic administration of COX-2 inhibitors can attenuate the immunosuppressive effects of UVB [25]. Additionally, PGE_2 injection has been shown to upregulate CXCL12 in lymph nodes [73]. Finally, it has been shown using small molecule agonists and antagonists that PGE_2 receptor EP4 is necessary for the immunosuppressive effects of UV light [131].

VI. Summary, recent work and current hypothesis

Studies in the field, including our own, have demonstrated that various environmental oxidative stressors are immunosuppressive. While mechanistic detail of this process is lacking, a major contributor to this immunosuppression is the activation of

the PAFR. It was recently found that the immunosuppressive effects of UVB can enhance melanoma tumor growth in vivo and that this effect is PAFR-dependent [25]. Thus, I suspect that pro-oxidative melanoma therapies, in particular chemotherapeutics, such as melphalan and etoposide, could have similar effects due to their nature as oxidative stressors. Thus, this work has two aims. The first aim is to investigate the possible role of PAF in chemotherapy-induced immunosuppression. The second aim is to further illuminate the mechanism of PAF-induced immunosuppression. The work in Chapter 3-I was carried out to determine if PAFR agonists could be made at high-dose chemotherapy in ILP chemotherapy. I hypothesized that ox-GPCs generated by IPL chemotherapy meadiate systemic immunosuppression leading to greater tumor growth of a second tumor.

Preliminary studies demonstrated that MC-deficient mice were resistant to PAF-induced increases in tumor growth. Furthermore, MCs have been shown to be necessary for UVB-induced immunosuppression [68]. Thus the second aim of this work, in Chapter 3-II, is to investigate the role of the MC in PAF-induced systemic immunosuppression and the mechanism by which MC mediate effects on the immune system. I hypothesize that the MC PAFR is necessary for PAF-induced immunosuppression and that MC PAFR activation promotes systemic immunosuppression in a process involving MC-derived mediator release.

Chapter 2: Materials and Methods

I. Chemotherapy induces systemic immunosuppression via the generation of PAFR agonists

A. Reagents and cells

Chemicals were purchased from Sigma-Aldrich unless indicated otherwise. Anti-CD25 and isotype antibodies were from BioXcell (West Lebanon, NH). SK23MEL and B16F10 melanoma cells were obtained from ATCC and were grown in DMEM high glucose supplemented with 10% fetal calf serum as described previously [25]. Plated cell lines were allowed to proliferate to ~80% - 90% confluency in 10 cm petri dishes, and washed with Hank's Balanced Salt Solution (HBSS) three times, then incubated with 2 mL of warm (37°C) HBSS containing 10 mg/mL fatty acid-free BSA and 2 μmol/L of serine hydrolase inhibitor pefabloc. Before the addition of chemotherapeutic agents or DMSO (0.5%) vehicle, in some experiments, cells were preincubated with antioxidants for 60 minutes. Incubations were quenched by adding of 2 mL of methanol (0°C) followed addition of dichloromethane, and then lipids were extracted as described [24, 26, 132].

B. Mice

Wild-type female C57BL/6 (PAFR-expressing; age 6–8 weeks) were obtained from the Charles River Laboratories. C57BL/6 age and gender matched PAFR–deficient (PAFR KO) mice were used for experiments and, generated as previously described [35], were obtained from Prof. Takao Shimizu (University of Tokyo Department of Biochemistry, Tokyo, Japan). FoxP3-EGFP reporter mice [133] that were obtained from JAX, and FoxP3-EGFP mice that were crossed with PAFR KO mice were also used in

some experiments. No difference was observed between PAFR KO and PAFR KO Foxp3-EGFP mice in these experiments. Immunodeficient NOD.CB17-PrkdcSCID/J (Common name: NOD SCID) mice were obtained from the Indiana University Simon Cancer Center Core facility. In antioxidant experiments, mice were given vitamin C-enriched chow (10 g/kg; Research Diets, Inc.) and bottles of 5 mM N-acetylcysteine (NAC) in water ad libitum for 10 days before and during intratumoral chemotherapy injections, until the end of the experiment similar to previous studies [25, 132]. All mice were housed under specific pathogen-free conditions at the Indiana University School of Medicine. All procedures were approved by the Animal Care and Use Committee of Indiana University School of Medicine.

C. Measurement of PAFR agonists

The presence and concentrations of PAFR agonists in lipid extracts from treated melanoma tumors/cells or IPL perfusates were measured by measuring intracellular Ca^{2+} mobilization in response to treating PAFR–expressing KBP cells and PAFR-null KBM with isolated lipid extracts, as previously described [130, 132]. In brief, KBP and KBM cells were incubated with a cell permeable fluorescent Ca^{2+}-sensitive indicator, fura-2-AM (4 μmol/L in HBSS without dye) for 90 minutes at 37°C. Cells were then washed, and resuspended in room temperature HBSS before use. Lipid extracts from treated cells/tumors, or ILP perfusates were added to Fura-2-loaded cells (1.0–1.5 X 10^6 cells/2 mL) in a cuvette at 37°C under constant stirring. Volume of lipid extracts used to treat the cells was normalized to cell number, wet tissue weight, or 1/10th volume of perfusate. 1μM CPAF and endothelin-1 (ET-1), dissolved in ethanol, were used as positive controls. Fura-2-AM fluorescence was measured in a Hitachi F-4010 spectrophotometer using

excitation and emission wavelengths of 331 and 410 nm, respectively. The influx of Ca^{2+} in cell suspensions was calculated as described previously [24, 130, 132] and plotted as percentage of maximal calcium flux induced by either CPAF or ET-1.

D. Mass spectrometry studies

Mass spectrometry analysis was accomplished in collaboration with Prof. Robert Murphy at University of Colorado, by Kathleen Harrison. Lipid extracts from treated cells/tumors or ILP perfusates were subjected to mass spectrometry analysis using the AB Sciex triple quadrupole QTRAP 5500 mass spectrometer, equipped with a CTC-PAL autosampler and a Shimadzu HPLC as previously described [134]. Please see Published Supplementary Materials and Methods for details of instrument settings and characterization of the various species monitored [135].

E. In vivo tumor growth studies

To determine whether intratumoral chemotherapy can affect melanoma tumor growth, 5 X 10^5 B16F10 cells, which inherently do not express functional PAFR [25], were injected subcutaneously into bilateral shaved dorsal hind flanks of both WT and PAFR KO mice to give rise to two tumors. Tumor growth was monitored by measuring length and width of tumors at daily with digital calipers (Mitituyo). Tumor volume was then calculated by the equation: (major length X minor length^2/2). Six days after tumor implantation and every third day thereafter, the tumor on the left flank was treated by injecting ~100 μL of either etoposide (36 mg/kg), melphalan (15 mg/kg), or PBS with 0.5% DMSO vehicle. The therapeutic dose of etoposide and melphalan was empirically derived by performing pilot studies in WT mice using different doses of chemotherapeutic (n=3–5). To determine whether COX-2 inhibitors can modulate the

tumor growth effects of chemotherapy, SC-236 (200 ng), NS-398 (5 μg), or 100 μL PBS with 0.5% DMSO vehicle were injected intraperitoneally, concurrently with intratumoral chemotherapy at day 0 and every 3 days afterwards.

F. Human isolated limb perfusion chemotherapy studies

In collaboration with surgeons Douglas S. Tyler and Paul J. Speicher from Duke University, as well as Christopher E. Touloukian at Indiana University, perfusate samples were obtained from subjects undergoing ILP regional melphalan chemotherapy for melanoma. During the procedure, aliquots of perfusate (8mL) were removed at various time points (after initiation of closed-circuit perfusion, after heating the core limb to 40°C, and 15, 30, 45 and 60 minutes following initiation melphalan treatment) from the extracorporal circulation and mixed with equal volumes of ice-cold methanol and dichloromethane, after which lipids were extracted. The human studies were approved by the Indiana University and Duke University School of Medicine Institutional Review Boards.

G. Statistical analysis

For all mouse studies, individual experiments contained at least four mice per experimental group and experiments were repeated at least once to verify reproducibility and to provide additional data for analysis. In collaboration with Indiana University statisticians Sandra K. Althouse and Susan M. Perkins, statistical differences were calculated using SAS Version 9.3. Tumor volume was calculated using the equation: (major length X minor length^2/2). For murine studies, analysis was focused on the end of the study around days 14 to 18, where available. Shapiro–Wilk and the Levene tests were used to check the normality of data and equal variances and were found to be a

reasonable assumption in all cases. For in vivo and in vitro data, equal or unequal variance t tests were used to compare differences between two groups. For comparing differences between more than two groups, two-way ANOVA tests (with Welch approximation if the variances between groups were unequal) and post hoc Tukey-adjusted pairwise tests. The data depicted represent mean values with SEM. Differences were considered statistically significant when the p value was less than 0.05 and trending but not significant when the p value was less than 0.10 but greater than 0.05.

II. Mast cell-derived histamine and prostaglandins mediate IL-33 receptor dependent PAF-induced immunosuppression

A. Reagents and cells

All chemicals were obtained from Sigma–Aldrich (St Louis, MO) unless indicated otherwise. COX-2 (NS-398 and SC-236) and TGF-β inhibitors (SB431542 and LY364947), and histamine EIA kit were obtained from Cayman Chemicals (Ann Arbor, MI). PGE_2 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 western were obtained from Abcam (Cambridge, MA). 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 mast cell populations (FcεR+, 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 1mL of 10% FBS containing IMDM media supplemented with 10 ng/mL of IL-3 in 12 well plates.

B. Mice

C57BL/6 (WT; Charles River Laboratories, Sulzfeld, Germany) and C57BL/6 *Kit*$^{Wsh/Wsh}$ (Wsh; The Jackson Laboratory, Bar Harbor, ME) mice were obtained commercially. PAFR KO (from Prof. T. Shimizu, University of Tokyo, Tokyo, Japan) and 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 mast cell transplantation were kindly provided by Dr. Garret FitzGerald (University of Pennsylvania, Philadelphia, PA). BoyJ mice were provided by the In Vivo Therapeutics Core (Indiana University School of Medicine, Indianapolis, IN). All mice were housed under specific pathogen-free conditions at the Indiana University School of Medicine. All procedures were approved by the Animal Care and Use Committee of Indiana University School of Medicine.

C. MC PAFR functional assay

The presence of PAFR on BMMCs was determined by the ability of CPAF to induce an intracellular Ca^{2+} mobilization response in BMMCs. In brief, BMMCs were preloaded with the Ca^{2+}-sensitive indicator, fura-2-AM (4 μmol/L in HBSS without dye) at 37°C for 90 minutes, washed, and resuspended in HBSS at room temperature before use. CPAF was then added to an aliquot of these cells (1.0–1.5 10^6 cells/2 mL) in a cuvette at 37°C with constant stirring. Fura-2-AM fluorescence was monitored in a Hitachi F-4010 spectrophotometer with excitation and emission wavelengths of 331 and 410 nm, respectively. The Ca^{2+} influx in suspensions was calculated as described [24, 130, 132].

D. Mast cell transplantation and contact hypersensitivity assay

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 Wsh PAFR KO mice. After 6 weeks post-implantation, mice were used for experiments. For contact hypersensitivity, mice were either treated on shaved dorsal skin with vehicle, UVB (7.5 kJ/m^2), histamine (200 μg s.c.), or CPAF (200 ng i.p.). Five days post-treatment 25μL of 0.5% DNFB (in 4:1 acetone/olive oil) was painted on shaved dorsal skin of mice. Nine days later ears were measured, and one ear was treated with 10μL of 0.5% DNFB while the other ear was treated with vehicle. After 24 hours, ear thickness was measured in these mice. The difference in ear swelling between DNFB and vehicle treated ears was normalized to the ear swelling in WT mice treated with vehicle.

E. 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 [136]. MC numbers were quantified by counting ten high power fields (HPF, 600X).

F. qRT PCR

Total RNA was extracted from treated mast cells using the RNAeasy 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*, *Hdc*, *Cxcr4*, *Il1rl1*, and *Tgfb1* against

Gapdh as the endogenous control using the ΔΔCt method on a Step One Real-Time PCR machine (Applied Biosystems, Foster City, CA). Each assay was performed in triplicate in a 10 uL reaction volume with Taqman Master Mix (SA Biosciences, Frederick, MD), 1 ng cDNA, primers at 500 nM and probe at 250 nM.

G. 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 concentrations of conjugated primary antibody for 30 minutes. Cells were then washed and resuspended in PBS to be stained with viability dye (eFluor 780) at a concentration of 1:1000 when applicable. For intracellular staining, unstimulated cells (unless otherwise noted) were fixed with IC Fixation buffer (#00-8222-49) or Fix/Perm Buffer (#00-5523-00) overnight, then washed and resuspended in Permeabilization Buffer (#00-8333-56). Cells were then stained with primary conjugated antibodies in Perm buffer for 30 minutes RT. Cells were then washed in Perm buffer and resuspended in FACS buffer. Cells were then resuspended to a concentration of about 10^5/100uL of cells in FACS buffer for analysis on the Invitrogen Attune Cytometer or the BD LSR II.

H. Western Blotting and Densitometry

Cells ($6x10^6$) were harvested and washed twice with PBS. Cell pellets were then lysed with 150 uL of lysis buffer (1% Triton-X, 10mM Tris base, 150mM NaCl, 1% protease inhibitor mixture (Sigma); pH 7.4) and centrifuged for 15 minutes at 14K rpm at 4°C. 120 uL of the supernatants were collected and their protein concentrations were measured by BioRad spectrophotometry methods. 30-100ug of protein with β-

mercaptoethanol-containing reducing SDS sample buffer was then loaded into a 10% acrylamide gel and run for 45 minutes under a constant 400mAmps. Proteins were then transferred onto a nitrocellulose membrane for 1hr at a constant 100V at 4°C. The membrane was then cut and incubated with blocking buffer (5% powdered milk, 0.1% Tween-20, in PBS) for 2hrs RT or overnight at 4°C. Membranes were then incubated with the appropriate amount of antibody for 2 hr RT or overnight at 4°C. Membranes were then washed with TBST (20mM Tris, 150mM NaCl, 0.1% Tween-20) four times for 5 minutes each before incubation with HRP-conjugated secondary antibody (1:5000) for 30 min 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.

I. ELISA

Cells ($3x10^6$) were plated and treated for the described length of time. Supernatants were then collected from suspensions centrifuged at 400g for 10 minutes. For intracellular lysates, cell pellets were washed twice with PBS and then resuspended in 1mL of deionized water. This suspension was then frozen in liquid nitrogen and thawed in 56°C water two times. Samples were then centrifuged at 14K rpm to remove any particulates. These supernatants were then diluted according to the detectable range of the ELISA (usually 1:10 or 1:100) in media or analysis buffer.

J. Statistics

For all murine studies, individual experiments were performed using at least four mice per experimental group and repeated as necessary (at least once) to verify reproducibility and provide additional data for analysis. All statistical calculations were performed using Prism 6. For in vivo and in vitro data, one-way ANOVA (with the Bonferroni or Dunnett correction for comparing means to all other means or means to vehicle, respectively) was used to compare one group and two way ANOVA (with the Holm-Sidak correction to compare treatment means to vehicle) was used to compare multiple groups. The data represent mean values with SEM. Differences were considered statistically significant when the P value was less than 0.05.

Name	Target	Supplier	Catalog #	IC50 (μM)
NS-398	COX-2	Tocris	0942	3.8
SC-236	COX-2	Tocris	3919	0.005
SB431542	TGFβR1	Cayman	13031	0.094
LY364947	TGFβR1	Cayman	13341	0.059

Table 1. List of inhibitors

Antibody	Supplier	Catalog #
Fc Block	BD Biosciences	553142
CXCR4-PE	eBioscience	12-0453-81
FceR-PE	eBioscience	16-5898-82
CD117-APC	eBioscience	17-1171-83
TGFb/LAP-PE	eBioscience	12-9821-80
CD25	BioXcell	BE0013
CD25	BioXcell	BE0012
HDC	Abcam	ab37291
COX-2	Abcam	ab62331

Table 2. List of antibodies

Gene	Supplier	Assay ID
Ptgs2	IDT	Mm.PT.56a.14196835
Hdc	IDT	Mm.PT.58.30065020
Cxcr4	IDT	Mm.PT.58.41597935
Tgfb1	IDT	Mm.PT.56a.11254750
Il1rl1	IDT	Mm.PT.58.11610831.g
Gapdh	Applied Biosystems	4352932E

Table 3. List of Taqman Primer Assays

COX-2 KO Primers	Supplier	Sequence
Forward	IDT	TGA GGC AGA AAG AGG TCC AGC CTT
Reverse	IDT	TTT GCC ACT GCT TGT ACA GCA ATT

Table 4. List of genotyping Primers

Chapter 3: Results

I. Chemotherapy induces systemic immunosuppression via the generation of PAFR agonists

A. Melanoma cells treated with chemotherapeutics generate PAFR agonists

Due to the fact that PAF is a lipid-derived molecule, which was first described as an activity having the ability to elicit platelet aggregation, measuring PAF concentrations or characterizing ox-GPCs is not straightforward. Thus, both a quantitative assay and a functional assay can be used to measure PAFR agonist concentrations. The quantitative assay is mass spectrometry through which, by the help of our collaborator Prof. Robert Murphy at University of Colorado, particular PAFR agonists in a sample can be characterized and quantitated. A Ca^{2+} mobilization assay can also be used as a functional read out to determine the ability of samples to activate the PAFR. This method was first developed in 1998 [130]. In short, a PAFR-negative human epidermal cell line (KB) was stably transduced with either the human PAFR to form KBP cells, or control MSCV2.1 vector to form PAFR-null (KBM) cells. Binding studies using radiolabeled PAFR antagonist WEB2086 revealed the presence of PAFR protein. Further characterization of these two cell lines demonstrated that treatment with CPAF would induce intracellular Ca^{2+} release and IL-8 production in KBP cells that can be measured by fluorimetry and ELISA, respectively. To measure intracellular Ca^{2+}release, cells are loaded with Fura-2-AM for 2 hours and fluorescence is measured immediately after treatment using a spectrofluorimeter [130].

Thus, to determine whether chemotherapeutics generate PAFR agonists in melanoma treatment, B16F10 melanoma cells were incubated with various

chemotherapeutics currently used for the treatment of melanoma for one hour. Lipid extracts were isolated from these cells, which contain any generated PAF and ox-GPCs, using standard organic separation techniques [137]. To test for the presence of any PAFR agonists in these lipid extracts, Ca^{2+} mobilization in Fura-2 loaded KBP and KBM cells was measured in response to treating the cells with aliquots of lipid extracts. Lipid extracts from B16F10 cells treated with etoposide activated calcium release in KBP (Figure 7A), but not KBM (Figure 7B) cells, suggesting the presence of PAFR-agonists in these extracts. Figure 7A shows examples of Ca^{2+} measurements from KB cells treated with controls, or lipid extracts from vehicle or etoposide treated melanoma cells. Endothelin-1 (ET-1; 1 μM) was used as a positive control for the KBM cells. In a similar fashion, time course experiments were performed using dacarbazine, etoposide, cisplatin, and melphalan, over the course of four hours. The fluorescence peak heights were compared to maximal Ca^{2+} response generated by 1 μM CPAF to derive % maximal values, to allow this assay to be semi-quantitative. Maximal calcium release in KBP cells induced by lipid extracts derived from B16F10 melanoma cells treated with chemotherapeutic agents was observed around two hours of incubation of cells with chemotherapeutic agents (Figure 7C). Similar results were observed with the human melanoma cell line SK23MEL (Figure 7D).

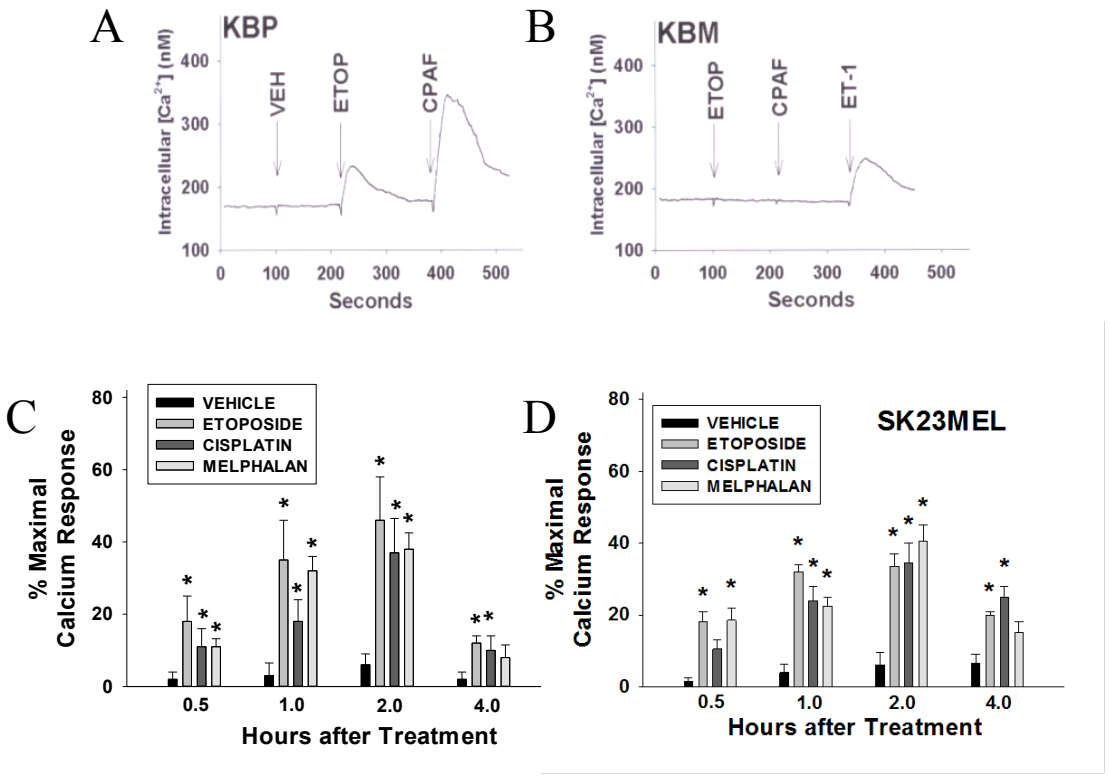


Figure 7. Etoposide, cisplatin, and melphalan induce the generation of PAFR agonists.

A and B. 5x10^6 B16F10 cells were treated with 100 µg/mL etoposide or DMSO vehicle for 1 hour. Cells were homogenized and lipids were extracted by organic separation. KBP (PAFR-expressing, **A**) or KBM (PAFR-null, **B**) cells were incubated with Fura-2 AM and treated with lipid extracts. Intracellular Ca^{2+} levels were monitored over time by measuring fluorescence. Excess CPAF or endothelin-1 (ET-1) (1 µM) was finally added to quantitate maximal Ca^{2+} response. **C and D.** 5x10^6 B16F10 (**C**) or SK23MEL (**D**) cells treated with 100 µg/mL of chemotherapeutic agents or 0.5% DMSO vehicle for various time points. Lipid extracts from cell lysates were tested for PAFR agonistic activity using Fura-2-loaded KBP cells. Graphs show the mean ± SEM percentage of maximum intracellular calcium release induced by 1 µM CPAF from at least four separate experiments. * denotes statistically significant ($p < 0.05$) changes in PAFR agonistic activity from control values for **C** and **D**.

Melanoma cells from patients have been shown to express PAFR, which can contribute to metastasis [31]. However, previous studies revealed that murine B16F10 melanoma cells do not express functional PAFRs as shown by lack of mRNA and failure to respond with an intracellular calcium mobilization response to CPAF [25]. To determine whether melanoma PAFR expression could modulate the generation of PAFR agonists by chemotherapy, the PAFR (*Ptafr*) gene (B16P) or MSCV2.1 retrovirus control vector (B16M) was previously transduced into B16F10 melanoma cells [25]. This way, PAFR expressing melanoma cells, B16P, induced greater amounts of PAFR agonists following treatment with dacarbazine, etoposide and cisplatin (Figure 8A). This finding suggests that PAFR expression on melanoma could contribute to the generation of PAFR agonists by chemotherapy. This is likely due to ox-GPCs acting on the melanoma cell PAFR to generate PAF enzymatically. To determine whether the PAFR agonists generated from chemotherapy-treated cells was due to the oxidation of cell membrane lipids, cells were pretreated with or without antioxidants, vitamin C (Vit C) and N-acetylcysteine (NAC), followed by treatment with chemotherapy or vehicle. Lipid extracts from B16P and B16M cells pretreated with antioxidants before treatment with cisplatin and etoposide were found to mobilize less calcium in KBP cells (Figure 8B).

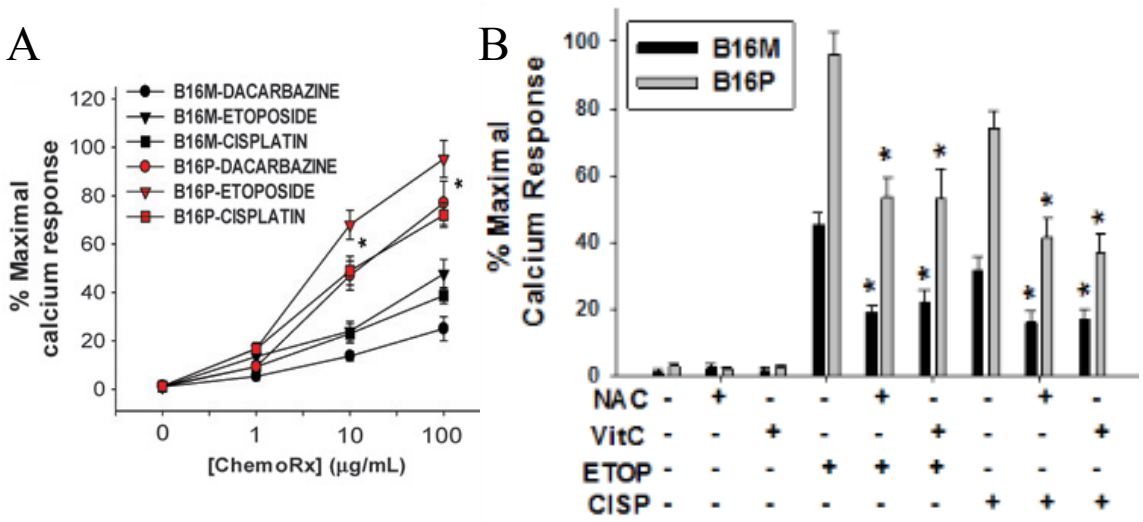


Figure 8. Generation of etoposide- and cisplatin-induced PAFR agonists is blocked by antioxidants and augmented by PAFR expression.
A. PAFR-expressing B16F10 PAFR cells (B16P) or PAFR-null B16F10MSCV2.1 (B16M) cells were treated with increasing doses of etoposide, dacarbazine, and cisplatin for 1 hour. Lipid extracts were isolated and then tested for the ability to activate the PAFR. The data are the mean ± SE percentage of maximum intracellular calcium response (normalized to CPAF) from three to four separate experiments. **B**. B16P and B16M cells were preincubated vitamin C (2.5 mM), NAC (5 mM), or 0.5% DMSO vehicle one hour prior to a one hour treatment with 100 µg/mL of etoposide or cisplatin. Lipid extracts were then isolated and tested for PAFR agonistic activity. The data are the mean ± SEM percentage of maximal intracellular calcium response (normalized to CPAF) from at least three separate experiments. * denotes statistically significant ($p < 0.05$) changes in PAFR agonistic activity from control values for A, and differences between chemotherapy-treated B16P and B16M cells in B. For C, the significant changes were for cisplatin at 10 µg/mL and for cisplatin, and etoposide at 100 µg/mL versus 0 dose.

These results suggest that chemotherapeutics induce the formation of PAFR agonists in part via the oxidation of resident lipids in treated melanoma cells. Lastly, to quantitate (Figure 9) and determine the structural identity (Table 5) of the PAFR agonists in the lipid extracts, collaborator Robert Murphy used mass spectrometry with deuterated internal standards using previously published methodologies [26]. They found that etoposide treatment generated greater than two-fold levels of PAF (1-O-hexadecyl-2-acetoyl-GPC), AzPAF (1-O-hexadecyl-2-azeleoyl-GPC), and BPAF (1-O-hexadecyl-2-butanoyl-GPC), among others, compared to vehicle treated cells. The structures of these PAFR agonists are summarized in Table 5. However, levels of the biologically inactive PAF precursor/metabolite lyso-PAF were unchanged following etoposide treatment. These in vitro studies demonstrate that chemotherapeutic agents, etoposide, cisplatin, and melphalan, induce melanoma cells to generate PAF and ox-GPCs with PAFR agonistic activity, in part due to ROS.

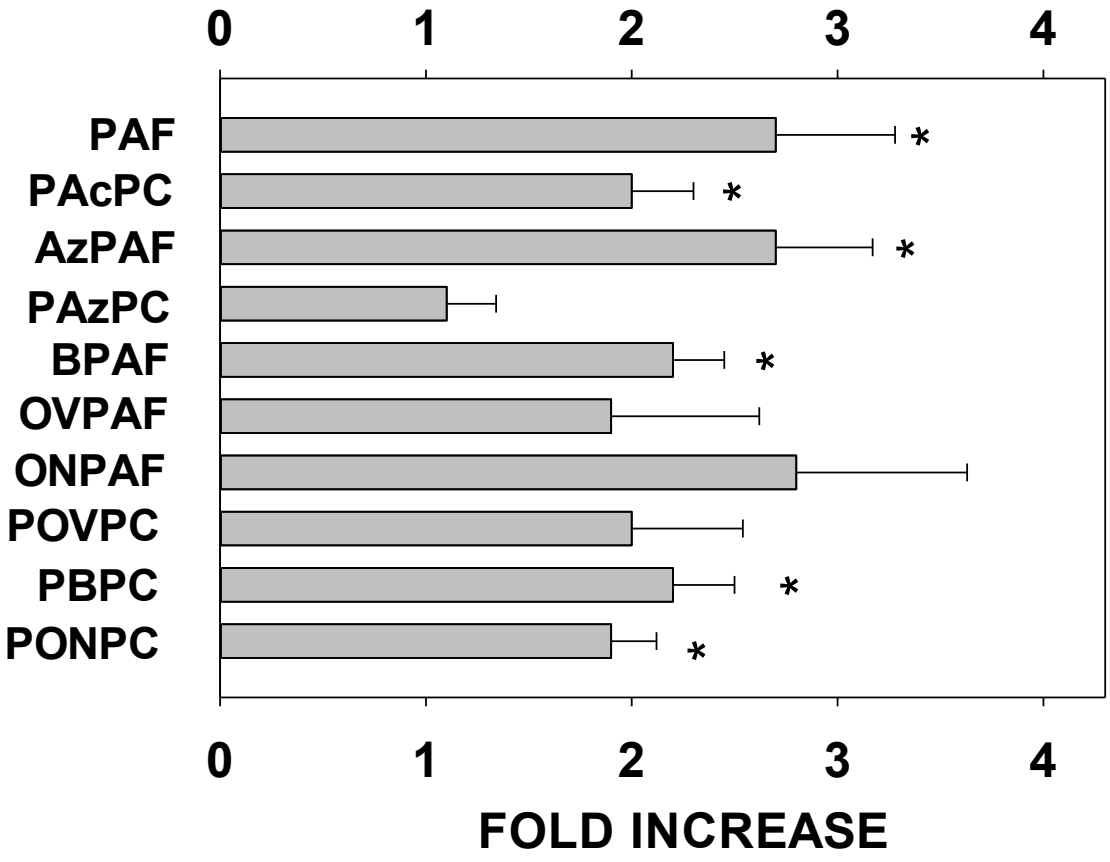


Figure 9. Structural characterization of etoposide-generated PAFR agonists. Lipid extracts from B16F10 cells treated with etoposide (100 μg/mL) or vehicle (0.5% DMSO) for 2 hours were collected. Lipid extracts were subjected to HPLC/MS/MS analysis. PAF and Ox-GPC species were quantified using deuterium-labeled internal standards. The data are expressed as mean ± SEM fold increase in PAFR agonist in etoposide over vehicle-treated. Representative of five separate experiments. * denotes statistically significant ($p < 0.05$) changes from vehicle-treated. Mass spectrometry analysis was kindly generated by Kathleen Harrison and Dr. Robert Murphy.

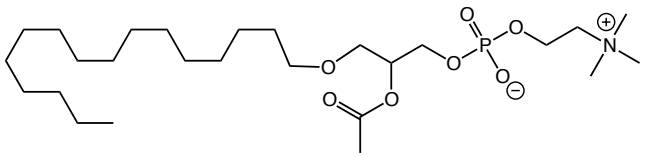
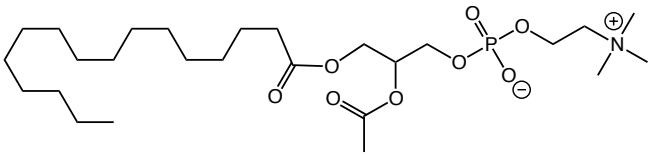
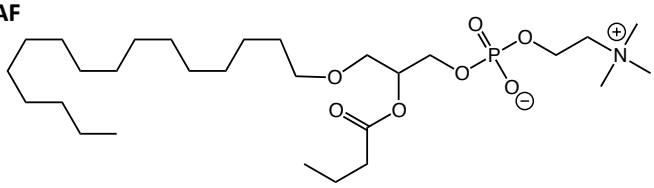
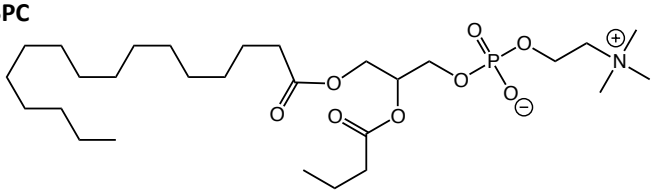
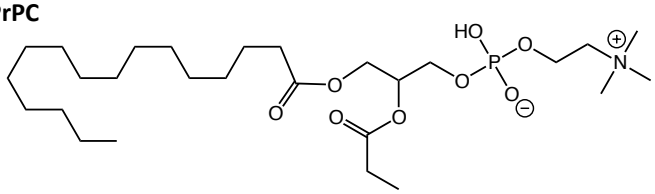
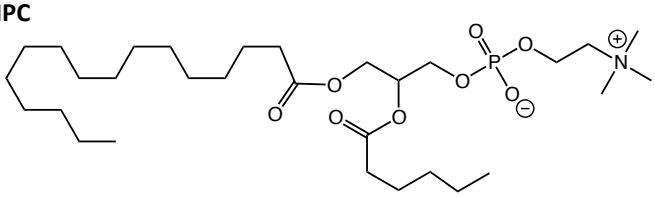
Glycerophosphocholine	Vehicle-Treated (pg/10^6 cells)	Etoposide-Treated (pg/10^6 cells)
PAF 	467 591 84 42 161 Mean 269	949 982 321 188 301 Mean 548
PAcPC 	1200 2320 5400 380 520 Mean 1964	3220 3490 12600 577 1453 Mean 4268
BPAF 	17.3 17.1 8.2 6.9 16 Mean 13.1	35.3 39.4 26.8 19.5 31.2 Mean 30.4
PBPC 	191 329 197 127 271 Mean 224	441 467 335 379 751 Mean 475
PPrPC 	3.8 8.4 0.1 3.2 8.4 Mean 4.8	4.9 19.9 2.8 7.8 10.5 Mean 9.2
PHPC 	1.4 3.8 1.3 7.2 11.3 Mean 5.0	1.5 4.7 3.7 34.1 39.6 Mean 16.7

Table 5. Glycerophosphocholine species generated in response to etoposide treatment of B16F10 cells. B16F10 cells were incubated for 2 hours with etoposide (100 μg/ml), or vehicle (0.5% DMSO) for 2 hours. PAF and Ox-GPC species from lipid extracts were quantified using deuterium-labeled internal standards. The table includes structures and amounts (pg per 10^6) of different GPCs in extracts from vehicle or etoposide-treated cells from 5 separate experiments. Mass spectrometry analysis was kindly generated by Kathleen Harrison and Dr. Robert Murphy.

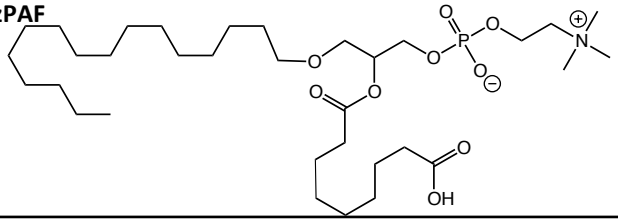
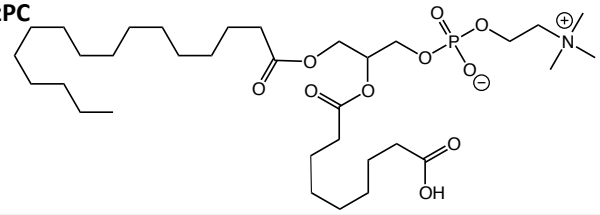
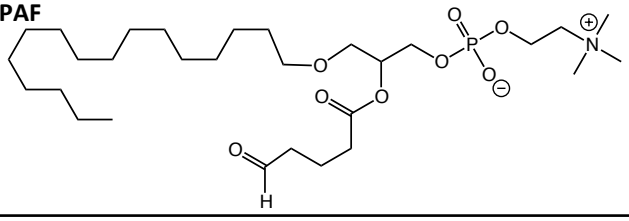
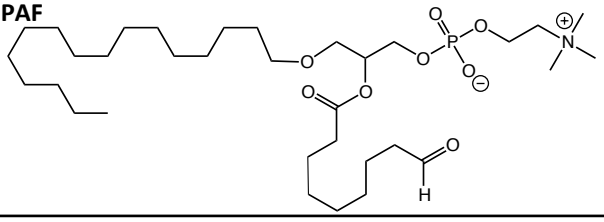
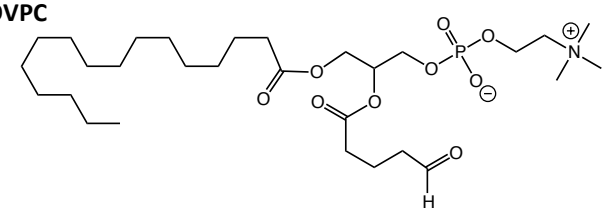
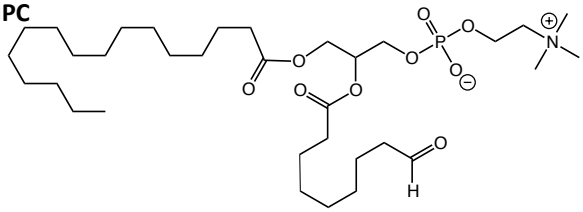
Glycerophosphocholine	Vehicle-Treated (pg/10^6 cells)	Etoposide-Treated (pg/10^6 cells)
AzPAF 	1.5 4.7 3.7 34.1 39.6 Mean 16.7	22.5 15.3 42.3 41.5 44.8 Mean 33.3
PAzPC 	219 97 139 362 251 Mean 214	140 77 179 336 497 Mean 246
OVPAF 	1.5 1.6 1.2 1.3 0.56 Mean 1.2	6.9 3.5 1.2 0.69 0.8 Mean 2.6
ONPAF 	1.9 1.3 1.3 0.18 0.75 Mean 1.1	8.8 4 5.9 0.29 0.25 Mean 3.8
POVPC 	3.4 1.5 6.5 4.2 4.3 Mean 4.0	7.2 6 8.6 4.2 5.7 Mean 6.3
PONPC 	23.1 16.8 23.8 8.2 6.7 Mean 15.7	56.4 35.2 47.4 9.4 11.3 Mean 31.9

Table 5. Glycerophosphocholine species generated in response to etoposide treatment of B16F10 cells. B16F10 cells were incubated for 2 hours with etoposide (100 μg/ml), or vehicle (0.5% DMSO) for 2 hours. PAF and Ox-GPC species from lipid extracts were quantified using deuterium-labeled internal standards. The table includes structures and amounts (pg per 10^6) of different GPCs in extracts from vehicle or etoposide-treated cells from 5 separate experiments. Mass spectrometry analysis was kindly generated by Kathleen Harrison and Dr. Robert Murphy.

B. Melanoma tumors treated with intratumoral chemotherapy produce immunosuppressive PAFR agonists

While systemic intravenous administration of chemotherapy is the most common route of administration, other routes of administration are useful in providing localized high-dose chemotherapy. IPL and intratumoral injections are available recourses to avoid the damaging effects of systemic high-dose chemotherapy on vital organs [99, 113, 114, 138, 139]. In order to ascertain whether chemotherapy generates PAFR agonists in vivo, an intratumoral injection approach was used to isolate the effects of drug on the tumor rather than the host. First, lipids were extracted from B16F10 tumors in mice one hour after intratumoral injection with either etoposide, melphalan, or vehicle. Lipid extracts from chemotherapy-treated tumors induced greater than a 10-fold increase in maximal calcium mobilization in KBP cells, compared to vehicle treated tumors (Figure 10A). Similar results were seen with human melanoma SK23MEL tumors transplanted into SCID mice (Figure 10B). Additionally, when the mice were put on an antioxidant diet (Vit C + NAC) before tumor implantation and chemotherapy (etoposide or melphalan) injection, the PAFR agonistic potential of the lipid extracts was diminished (Figure 10A).

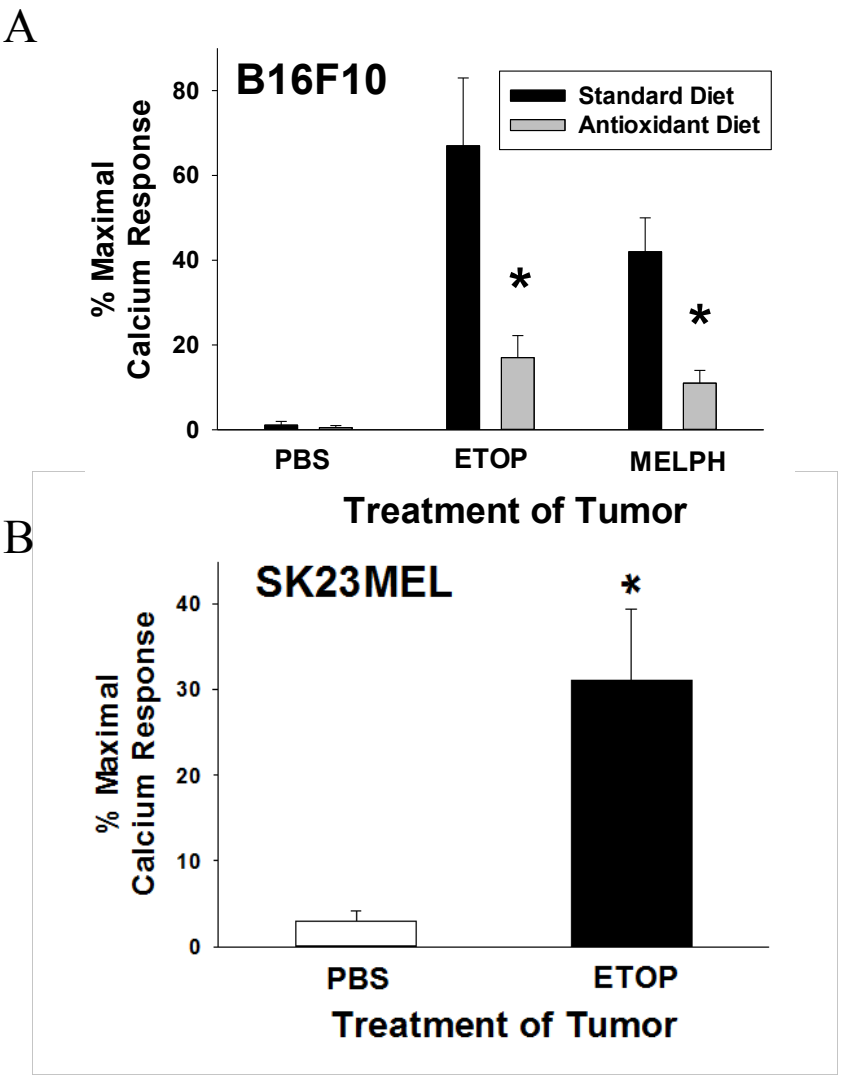


Figure 10. Intratumoral etoposide, and melphalan induces the generation of PAFR agonists.

A. WT mice were fed regular chow, or vitamin C-enriched chow (10 mg/kg) and NAC (5 mM) in water, ad libitum for 10 days before implantation of B16F10 tumors. **B.** SK23MEL tumors were implanted on SCID mice on a regular diet. **A** and **B**. Lipid extracts were obtained from 10 mg samples of tumors 1 hour following intratumoral injection with either etoposide (36 mg/kg), or melphalan (15 mg/kg), or PBS vehicle (100 µL). Lipid extracts were then tested by calcium mobilization for PAFR agonistic activity. The data are the mean ± SEM percentage of maximum intracellular calcium response (normalized to CPAF) from four to six separate tumors. * denotes statistically significant ($p < 0.05$) changes in levels of PAFR agonists in comparison to vehicle-treated tumors.

Since the generation of PAFR agonists was found in tumors following etoposide and melphalan chemotherapy injection, this observation led to the hypothesis that PAFR agonists could promote growth of a second tumor via systemic immunosuppression. Systemic administration of chemotherapeutics was found to be effective in reducing B16F10 tumor growth, but highly toxic to mice. Thus, to minimize toxicity in the mice and potential effects of chemotherapeutic agents on the immune system, a dual-tumor model was devised in which one tumor is treated with high-dose chemotherapy, while the contralateral tumor is measured over time. This dual-tumor model is not only a relevant model for intratumoral and IPL therapy in the clinic, but perhaps also a model of metastatic tumor growth. Using this model melphalan was found to have reduced efficacy in reducing growth of a second tumor in WT hosts compared to PAFR KO mice (Figure 11A). Notably, there was no discernable difference in growth of a second tumor between WT and PAFR KO mice when tumors were treated with vehicle (Figure 11B). This finding suggests that this difference in tumor growth may be PAFR-dependent.

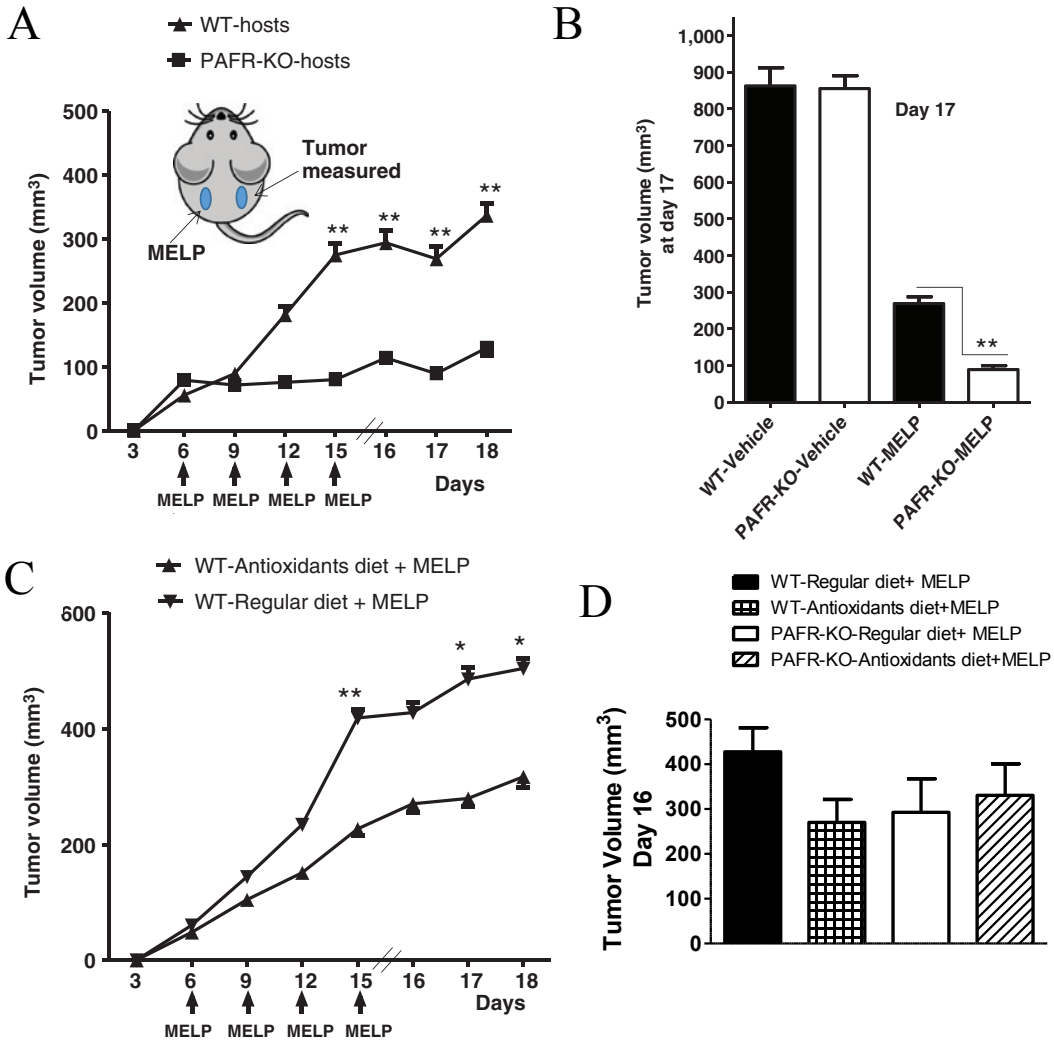


Figure 11. Intratumoral melphalan treatment augments the growth of untreated B16F10 melanomas in a PAFR–dependent manner.

B16F10 tumors were implanted on bilateral dorsal hind flanks in WT and PAFR-KO (*Ptafr-/-*) mice. **A-D.** Six days after implantation and every third day thereafter, the left tumor was injected with melphalan (15 mg/kg; n = 6-7; **A** and **B**), or vehicle (n = 5-6), while the contralateral tumor was undisturbed and measured. The data depicted are the mean ± SEM of tumor volume of untreated tumors over time. **B.** Data depicted is the tumor volume on day 17 from WT and PAFR-KO mice treated with intratumoral vehicle or melphalan. **C and D.** As in Fig. 8 mice were fed an antioxidant diet for 10 days before implantation of bilateral B16F10 tumors. Six days after implantation, one tumor from each mouse was injected with melphalan (n = 10–11) and every 3 days thereafter. The data depicted are the mean ± SEM of tumor volume of untreated tumors over time. **D.** Data represent the volume of untreated tumors at day 16 from melphalan-treated WT and PAFR-KO mice with regular or antioxidant diet. Statistical significance of changes in tumor volumes denoted by ** ($p < 0.01$); * ($p < 0.05$); # ($p < 0.1$).

In light of evidence suggesting that chemotherapy generated PAFR agonists via the oxidation of lipids in vivo, this evidence led to the hypothesis that ox-GPCs might also be responsible for the increased growth of a second tumor in WT mice. To test this hypothesis, mice were kept on an antioxidant diet (vitamin C + NAC) for 10 days before the implantation of tumors. Using a dual-tumor model, it was found that when one tumor was treated with melphalan, contralateral tumor growth in mice on antioxidants had significantly decreased tumor growth compared with those on a normal diet (Figure 11C and 11D). Similar, results were observed when tumors were treated with etoposide (Figure 12A and B). Notably, there was no difference in contralateral tumor growth from chemotherapy (melphalan or etoposide) treated tumors between PAFR KO mice given antioxidants and normal diet (Figure 13). These findings suggest that the promotion of growth of a second tumor was mediated by PAFR agonists produced by ROS in tumors treated with chemotherapy.

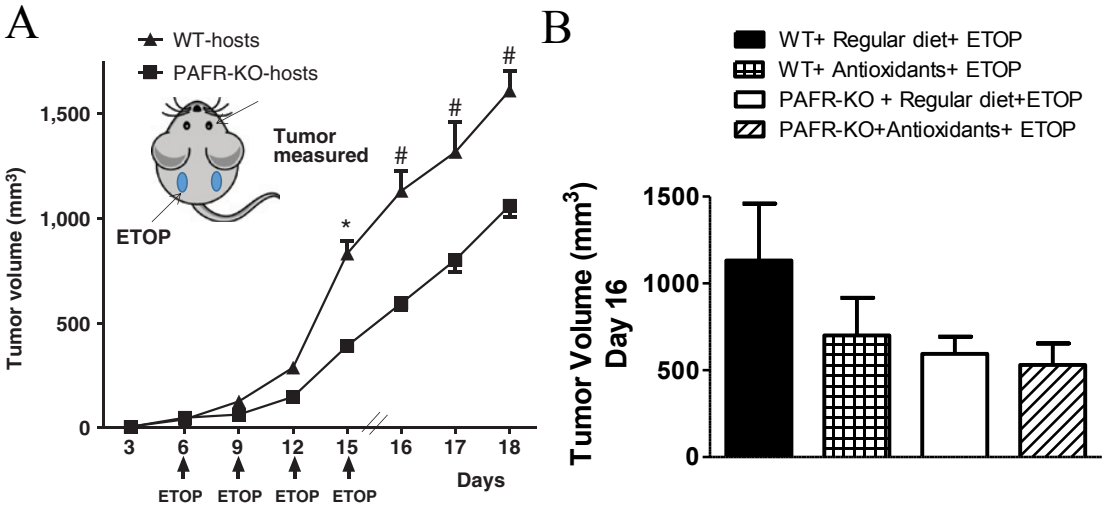


Figure 12. Intratumoral etoposide augments untreated tumor growth in a PAFR and ROS-dependent manner.

A. B16F10 tumors were implanted on bilateral dorsal hind flanks in WT and PAFR-KO (*Ptafr-/-*) mice. **A and B.** Six days after implantation and every third day thereafter, the left tumor was injected with etoposide (36 mg/kg; n = 5-6; **A** and **B**), or vehicle (n = 5-6), while the contralateral tumor was undisturbed and measured. The data depicted are the mean ± SEM of tumor volume of untreated tumors over time. **B.** Data represent the volume of untreated tumors at day 16 from etoposide-treated WT and PAFR-KO mice (n=9-12) with regular or antioxidant diet. Statistical significance of changes in tumor volumes denoted by ** ($p < 0.01$); * ($p < 0.05$); # ($p < 0.1$).

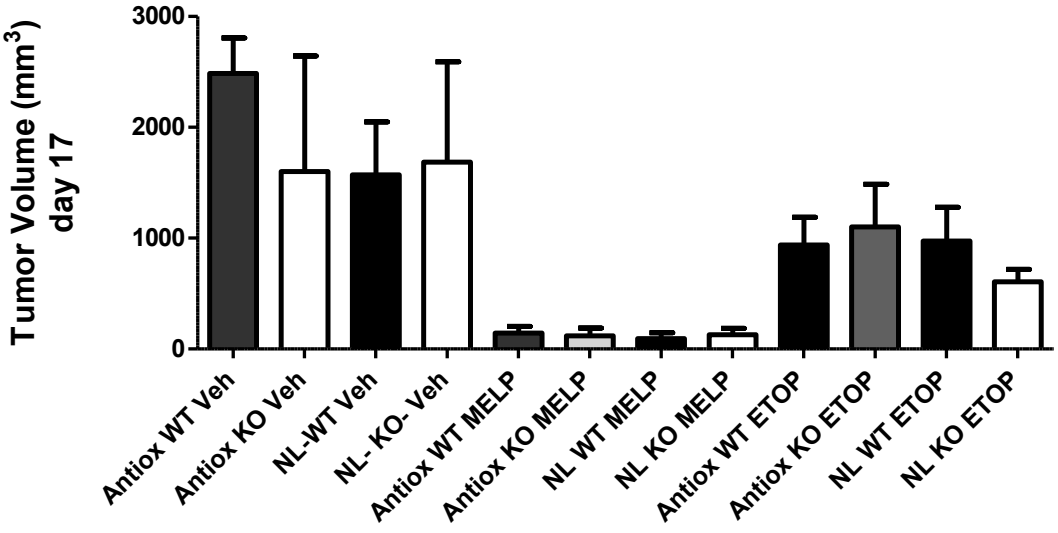


Figure 13. Growth of tumors treated with intratumoral melphalan or etoposide versus vehicle at Day 17.

As in Fig. 9 mice were fed an antioxidant diet for 10 days. B16F10 tumors were then implanted on bilateral dorsal hind flanks in WT and PAFR-KO (*Ptafr-/-*) mice. Six days after implantation, the left tumor from each mouse was injected with vehicle, melphalan (15 mg/kg; n = 9–11) or etoposide (36 mg/kg; n=9-12), and every 3 days thereafter. The data depicted are the mean tumor volumes ± SEM at Day 17 post-tumor implantation of tumors treated with chemotherapy, or vehicle, implanted on the left flank. Between WT & PAFR-KO mice, there were no statistically significant differences in the growth of chemotherapy- or vehicle-injected tumors.

There are significant data suggesting that COX-2 is involved in the immunosuppressive effects of UVB and PAF [16, 24, 25, 118, 130, 131]. Thus, this evidence led to the hypothesis that COX-2 was involved in mediating the increase in contralateral tumor growth by chemotherapy in a PAFR-dependent fashion. Similarly, it was hypothesized that CPAF administration could modulate tumor growth in a COX-2 dependent fashion. To test this hypothesis mice were treated with either intraperitoneal injections of vehicle or CPAF, and vehicle or COX-2 inhibitor SC-236, every 6 days after single tumor implantation on Day 0. By measuring tumors over time, it was found that CPAF i.p. promoted tumor growth (Figure 14A). While SC-236 did not modulate tumor growth alone, it did inhibit the tumor promoting effects of CPAF. Similar results were seen in using a structurally different COX-2 inhibitor, NS398 (Figure 14B). Noteworthy, however, is the fact that neither SC-236, nor CPAF, had any effect on tumor growth in PAFR KO mice (Figure 14C). Next to test the hypothesis that COX-2 inhibitors could block the tumor promoting effects of chemotherapy, a dual-tumor model was used, where mice were either treated with COX-2 inhibitor, SC-236, or vehicle every three days during intratumoral chemotherapy treatment. Tumor measurements over time showed that in mice treated with intratumoral chemotherapy, untreated tumors grew smaller and at a slower rate when mice were treated with COX-2 inhibitor SC-236 compared to mice given vehicle treatment (Figure 15A and 15B). These findings indicate that COX-2 inhibitors can block the growth promoting effect of chemotherapy on a contralateral tumor.

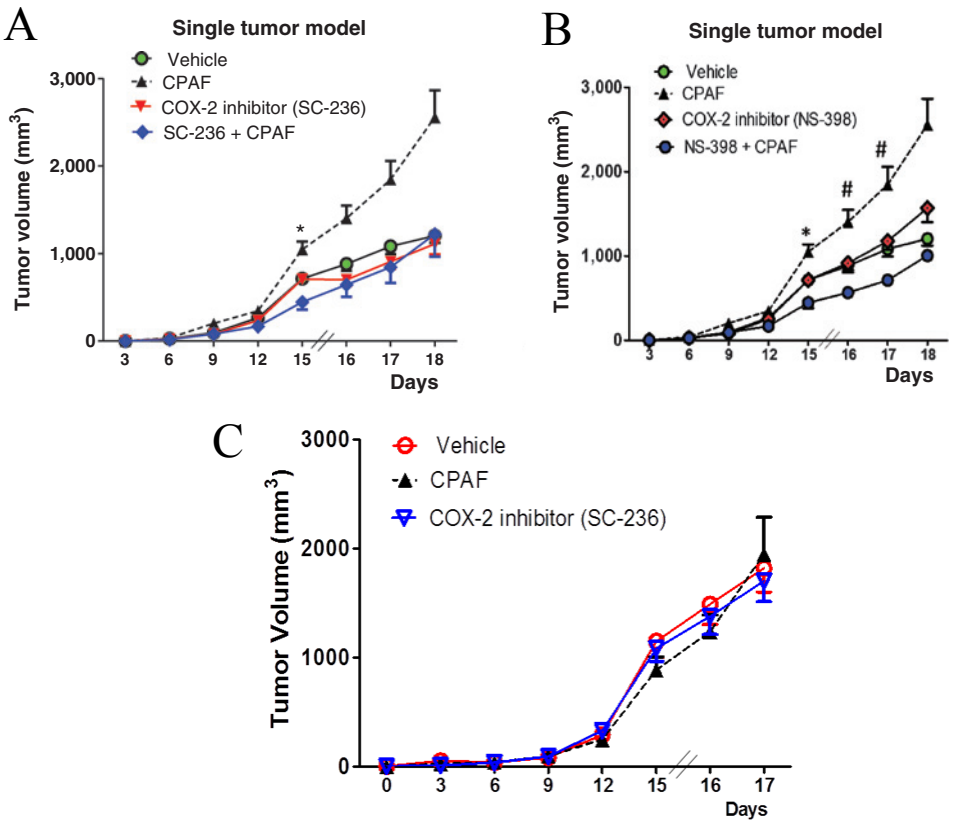


Figure 14. COX-2 inhibitors block PAFR-mediated augmentation in tumor growth.

A and B. A single B16F10 tumor was implanted on the dorsal hind flanks in WT mice (n = 6-7). Starting on the day of tumor implantation, the mice were treated every 6 days with CPAF (250 ng i.p.) or vehicle, and vehicle or COX-2 inhibitors SC-236 (200 ng i.p.; **A**), NS-398 (5 µg i.p.; **B**). **C.** A single B16F10 tumor was implanted on the dorsal hind flanks in PAFR-KO mice (n = 4-5). Mice were treated at tumor implantation and every 3 days with intraperitoneal injections of vehicle, CPAF (250 ng), or COX-2 inhibitor SC-236 (200 ng). Tumor growth was measured over time. The data depicted are the mean ± SEM of tumor volume. Statistical significance of changes in tumor volumes is denoted: ** ($p < 0.01$); * ($p < 0.05$); # ($p < 0.1$).

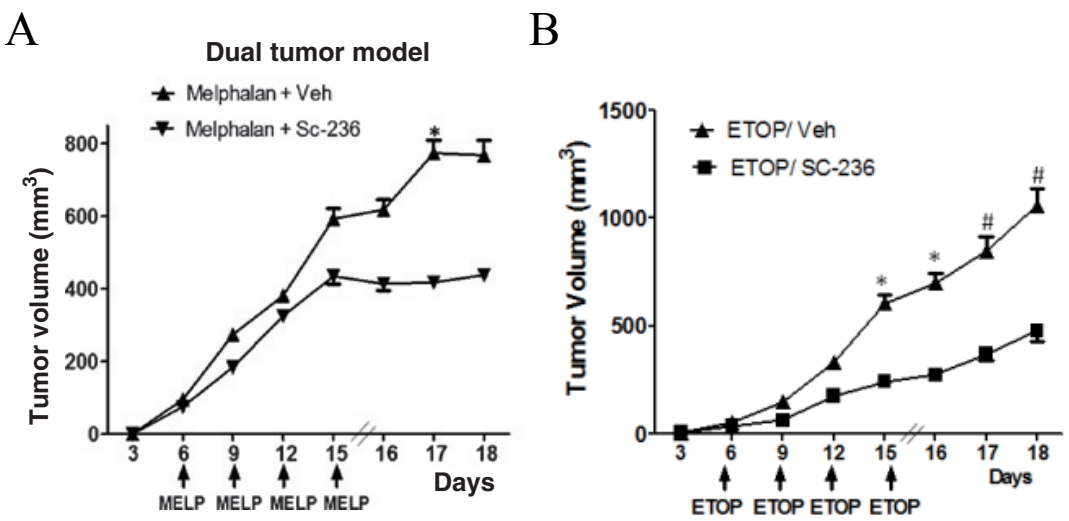


Figure 15. COX-2 inhibitors block melphalan- and etoposide-induced augmentation of tumor growth.

A and B. B16F10 tumors were implanted on bilateral dorsal hind flanks in WT mice. Mice were treated with COX-2 inhibitor SC-236 (200 ng i.p.) or vehicle every 3 days starting on the day of tumor implantation. Left tumors were also injected on day 6 post-tumor implantation with PBS vehicle (n = 7) or chemotherapy (melphalan (n = 16; **A**) or etoposide (n=12-14; **B**) and every 3 days thereafter . Untreated tumors were measured over time as in Fig. 11. The data depicted are the mean ± SEM of tumor volume of untreated tumors over time in which the contralateral tumor was treated with chemotherapeutic agent. Statistical significance of changes in tumor volumes is denoted: ** ($p < 0.01$); * ($p < 0.05$); # ($p < 0.1$).

There are several reports suggesting that Tregs are involved in the immunosuppressive effect of UVB and PAF [24, 25, 118, 119]. This information led to the hypothesis that Tregs could mediate the immunosuppressive effects of chemotherapy. To test this hypothesis, an α-CD25 depleting antibody was used to deplete Tregs [24, 25]. In a dual tumor model, it was found that depleting Tregs using an antibody approach protected against growth of the untreated tumor, when treating the contralateral tumor with etoposide (Figure 16D). These data support the hypothesis that Tregs might be involved in mediating the promotion of tumor growth by CPAF. To investigate whether generated PAFR agonists might induce the expansion of FOXP3+ Tregs, a FOXP3-EGFP reporter mouse was obtained from JAX [133]. Using flow cytometry, mice treated with CPAF were found to have increased populations of intratumoral Tregs nine days after CPAF injection in a process blocked by COX-2 inhibitors (Figure 16A and B). This increase in Treg numbers in tumors and draining LNs, however, could be attenuated by COX-2 inhibitor, SC-236, as measured by EGFP (*Egfp*) expression by qRT-PCR (Figure 16C).

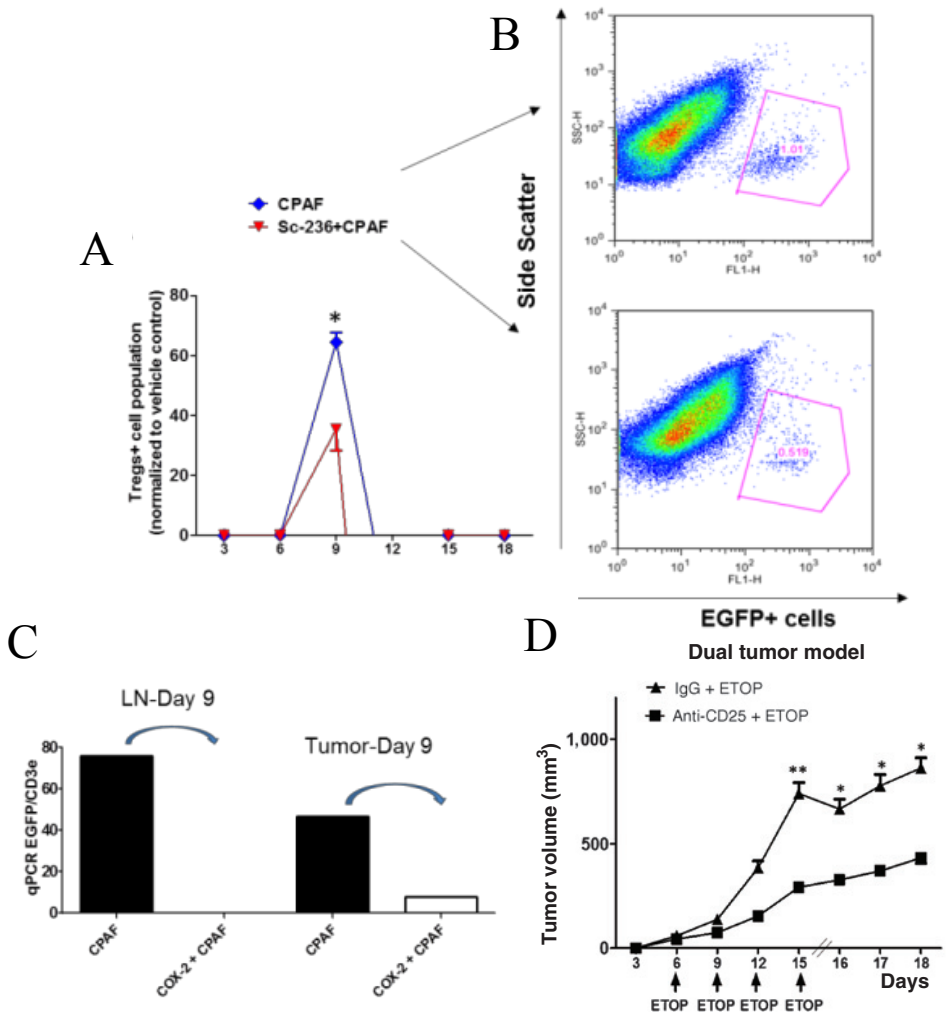


Figure 16. Tregs are necessary for etoposide-induced augmentation of tumor growth.

FoxP3^{EGFP} WT mice (n=6-9) were implanted with a single B16F10 tumor on dorsal hind flanks. Mice were then treated with with COX-2 inhibitor SC-236 (200 ng) or vehicle PBS (100µl) i.p. starting on day of implantation and every third day. On days 0, 6 and 12 mice were also treated with CPAF or vehicle. On days 6, 9, 12, 15 and 18, tumors were harvested and analyzed for EGFP+ cells by flow cytometry and qRT-PCR as a marker for Tregs. **A.** Mean percent increase in tumor EGFP+ populations, normalized to vehicle treated groups, between CPAF and SC-236 + CPAF treated groups over various time points. Error bars represent SEM. **B.** Representative dot plot for tumor EGFP+ cells 9 days after CPAF or SC-236 + CPAF treatment. **C.** Relative *Egfp* mRNA levels normalized to *Cd3e* in tumors from CPAF or SC-236 + CPAF treated mice. **D.** B16F10 tumors were implanted on bilateral dorsal hind flanks in WT mice. Two days prior to tumor implantation, groups of mice (n=7-8) were treated with isotype (IgG1 and IgM1) or anti-CD25 (clones PC61.5.3 IgG1 and 7D4 IgM1, 1 mg each). Tumors were injected with etoposide and contralateral tumors were measured as outlined in Fig. 11. Statistically significant changes are denoted: ** ($p < 0.01$); * ($p < 0.05$). Flow cytometry and qPCR for this figure were kindly contributed by Dr. Sahu.

C. Isolated limb perfusion chemotherapy generates PAF and PAFR agonistic ox-GPCs in humans

In light of evidence suggesting that PAFR agonists are generated as a result of chemotherapy in mice, it became relevant to study this phenomenon in humans. To test the hypothesis that high-dose chemotherapy in ILP generates PAF-like ox-GPCs, perfusate samples from human patients undergoing ILP melphalan chemotherapy for solid tumors (melanoma, sarcoma) were obtained in collaboration with surgeons Douglas S. Tyler and Paul J. Speicher from Duke University, as well as Christopher E. Touloukian at Indiana University. To measure PAFR agonists in the samples, lipids were extracted from the perfusate samples. Then the PAFR-Ca^{2+} biochemical assay was used to measure the ability of these lipid isolates to activate the PAFR. A basal concentration of PAFR agonists were measured from perfusate samples once the perfusion flow was established (Control) and did not change after the limb was heated to 40°C. However, a significant increase in PAFR activation was measured from perfusates collected 15-60 minutes after melphalan was introduced into the perfusate (Figure 17A). Collaborator Prof. Robert Murphy, University of Colorado, found by mass spectrometry analysis that various ox-GPCs were generated during this process. Of significance, production of PAF, ONPAF and AzPAF, were significantly increased in perfusates following melphalan administration compared to control perfusates (Figure 17B). These findings demonstrate that chemotherapy administered by IPL generates a significant quantity of PAFR agonists in the clinical setting.

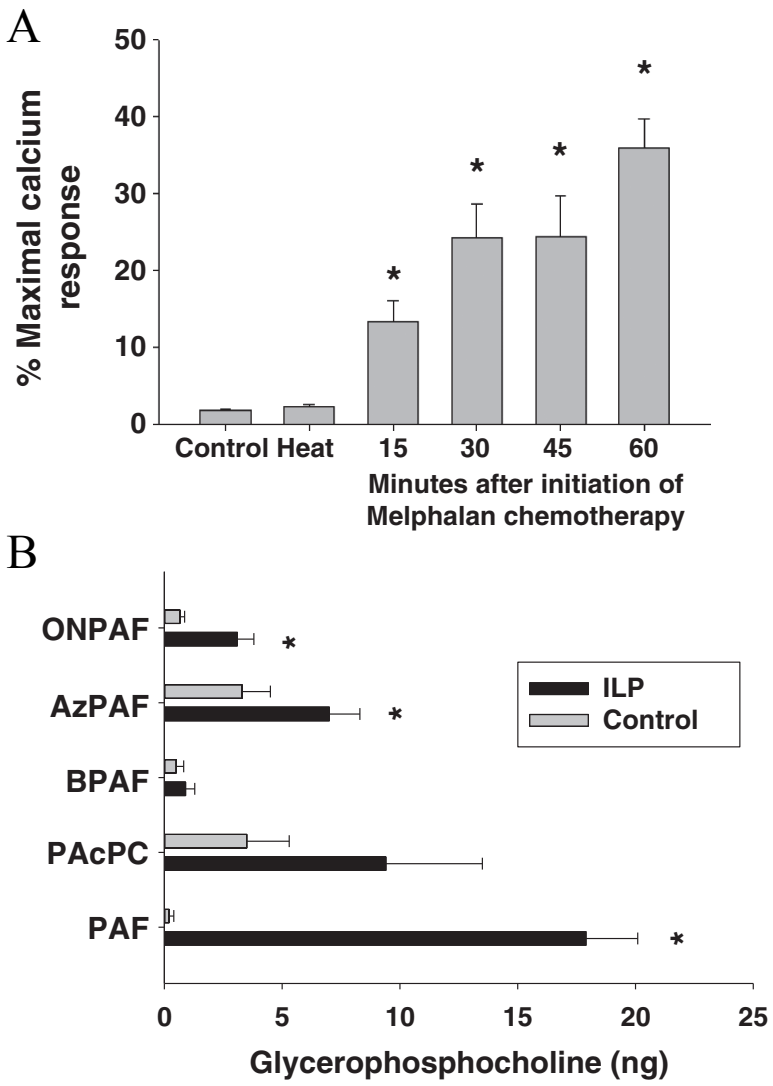


Figure 17. Isolated limb perfusion melphalan therapy generates PAFR agonists in humans.

A. ILP samples were drawn at various time points [once the perfusion circuit was established (Control), once the limb was heated to 40 °C (Heat), and 15, 30, 45 and 60 minutes after the initiation of melphalan chemotherapy] from the perfusate of six separate subjects undergoing high-dose chemotherapy. Lipid fractions were isolated from these perfusate samples and tested by calcium mobilization for PAFR agonistic activity as in Fig. 7. The data are the mean ± SEM percentage of maximum intracellular calcium release (normalized to 1 μM CPAF) of duplicate samples. **B.** Perfusates collected at two time points (Control and 30 minutes after chemotherapy) from three subjects were analyzed by mass spectrometry as in Fig. 9. The data depicted are mean mass (ng) of GPC ± SEM per 8 mL of perfusate from three separate subjects. * Denotes statistically significant ($p < 0.05$) changes from values measured in control perfusates. Mass spectrometry analysis was kindly generated by Kathleen Harrison and Dr. Robert Murphy.

D. PAFR agonist augmentation of tumor growth requires MCs

There are several reports that MCs are required for PAF- and UV-induced immunosuppression [68, 73, 74, 127, 140]. These reports led to the hypothesis that MCs may also be necessary for PAF-induced augmentation of tumor growth. To test this hypothesis, mast cell-deficient (Wsh) mice were transplanted with B16F10 tumors and the mice were treated with vehicle, or CPAF, on days 0, 6 and 12. Tumor measurements over 14 days demonstrated that there were no significant differences in tumor growth between mice treated with vehicle or CPAF (Figure 18). This evidence suggests that MCs are involved in the tumor promoting effects of PAF. The mechanism by which MCs are involved in this pathway remains poorly understood.

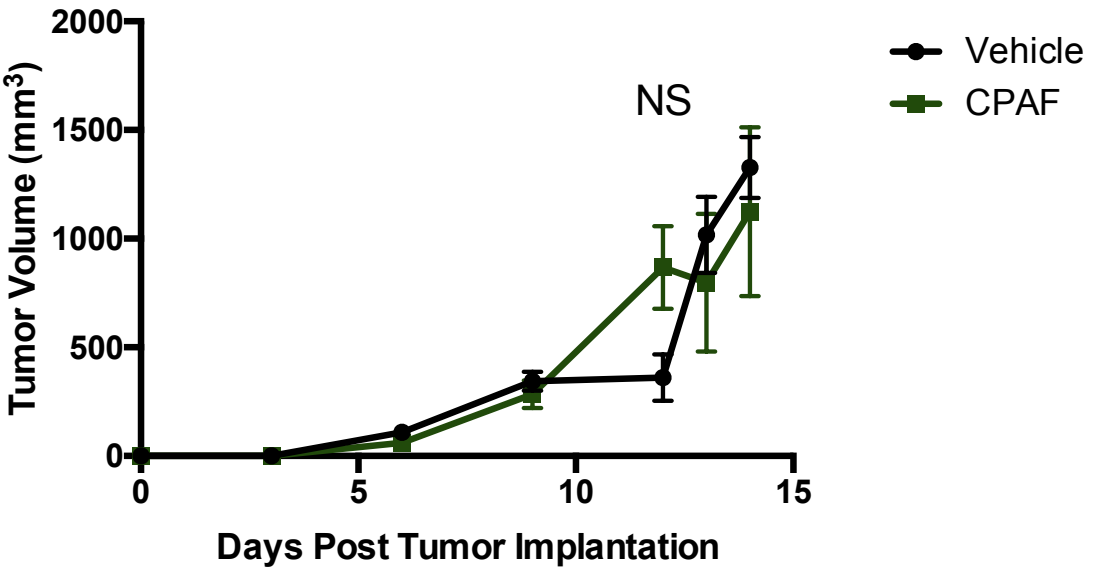


Figure 18. Tumor measurements in MC-deficient Wsh mice treated with intraperitoneal injections of CPAF vs vehicle.
Wsh mice (n=5) were implanted with a single B16F10 tumor and were treated at day 0 and every 6 days with intraperitoneal injections of CPAF (250 ng) or vehicle (PBS). Growth of untreated tumor was assessed over time as in Fig. 11. The data depicted are the mean ± SEM of tumor volume over time. Statistical significance determined using two-way ANOVA and using post-hoc Sidak multiple comparisons method for every time point, with alpha=5%.

II. Mast cell-derived histamine and prostaglandins mediate IL-33 receptor dependent PAF-induced immunosuppression

A. Validation of MC transplant CHS model

To elucidate the role of MCs in PAFR-mediated immunosuppression, a dinitrofluorobenzene (DNFB) contact hypersensitivity model (CHS) was used (Figure 19). This particular model is well established in the literature [141-144]. This allergy model is better suited than a tumor model to understand the role of PAF and its effects in the inflammatory process, for it has well-defined mechanisms. The first set of experiments sought to validate a MC transplant model for the use in CHS experiments. This model would allow us to study the role of a specific population of mast cells, the dermal mast cell. Bone marrow-derived mast cells (BMMCs) were used for this model, due to the fact that MC reconstitution into Wsh mice has been well described in the literature [69].

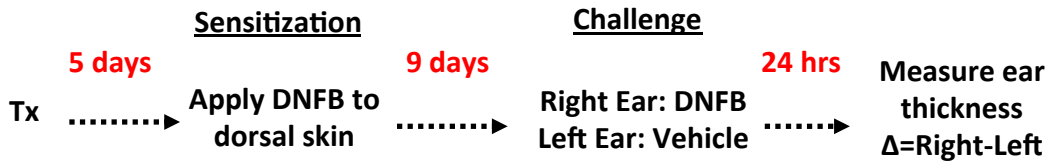


Figure 19. CHS model.
Mice are treated (Tx) with CPAF (250ng, i.p.) or vehicle (PBS, i.p.), histamine (200 µg s.c.) or UVB (7.5kJ/m^2) on shaved dorsal skin. Five days later mice are sensitized to DNFB by painting 25µL of 0.5% DNFB in acetone:olive oil (4:1) on shaved dorsal skin. Nine days later ears are measured, one ear of the mice is treated with 0.5% DNFB, whereas the other is treated with vehicle. Ear thickness is measured 24 hours later. Change in ear thickness is the difference in ear thickness between right and left ears. Change in ear thickness is normalized to WT vehicle-treated mice.

BMMC were obtained by flushing femurs and tibia from 8-12 week old mice and plating the cells obtained with IL-3 (10 ng/mL) supplemented media for 4 weeks (Figure 20A). Flow cytometry analysis revealed that >90% of the cells in culture were FcεRI+ ckit+, suggesting that the cells were mature mast cells (Figure 20B). These BMMC were then incubated with Fura-2-AM and subsequently treated with CPAF. BMMCs expressed a functional PAFR, due to the ability of Fura-2 loaded MCs to mobilize Ca^{2+} when treated with CPAF (Figure 20C). MCs (10^6 cells) were then transplanted into the dorsal skin of Wsh mice to reconstitute dermal MCs.

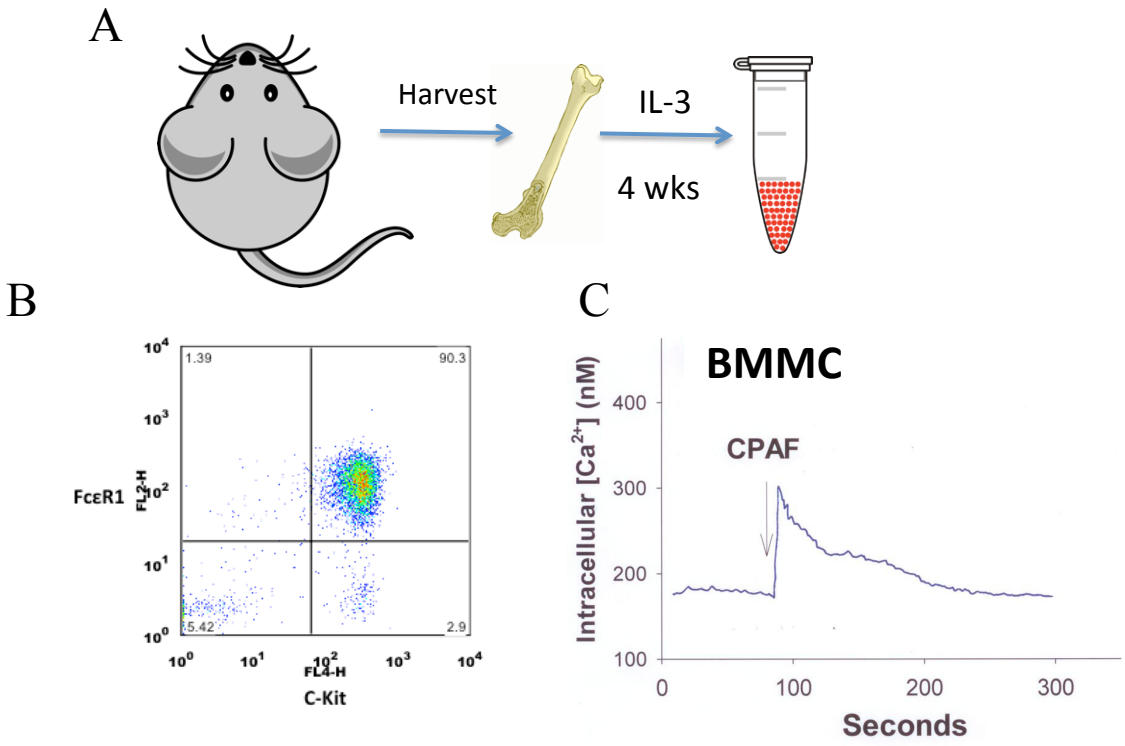


Figure 20. BMMCs express functional PAFR.
A. Mouse femur and tibia marrow were flushed with IMDM media. Mononuclear cells were isolated by Ficoll and plated in media supplemented with IL-3 (10 ng/mL) for 4-8 weeks. **B.** After four weeks, cells are analyzed for ckit and FcεRI expression by flow cytometry. Culture method yields >90% ckit+ FcεRI+ cells. **C.** Representative Ca^{2+} mobilization plot. After four weeks, cultured bone marrow cells were incubated with Fura-2 AM and treated with CPAF. Subsequent, calcium mobilization was measured by fluorimetry. Figures are representative of three experiments.

A CHS assay with WT, PAFR KO, Wsh and Wsh mice transplanted with MCs was performed that showed that CPAF and UVB irradiation attenuate the ear swelling response to DNFB challenge in WT mice, compared to vehicle treated mice (Figure 21). However, as depicted in Figure 21, PAFR KO and Wsh mice are insensitive to the immunosuppressive effects of CPAF/UVB. Interestingly, 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.

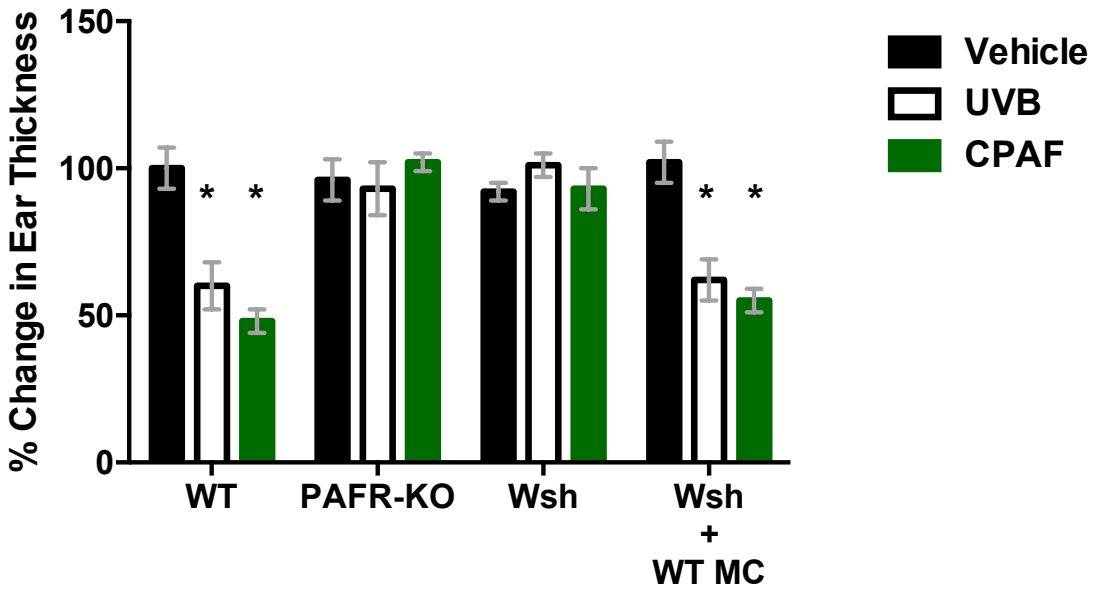


Figure 21. MC reconstitution rescues UVB and CPAF induced systemic immunosuppression in Wsh mice.
Groups of 8-12 WT, PAF-R KO, Wsh, or Wsh mice reconstituted with WT MC were treated with UVB (7.5kJ/m^2) , CPAF (250 ng) , or vehicle 5 days prior to DNFB sensitization. Mice were challenged with DNFB on Day 9 post-challenge and ear thickness was measured 24 hours later. * Denotes statistically significant differences ($p<0.05$) in ear thickness measurements normalized to vehicle treated mice. Statistical significance determined using two-way ANOVA and the post-hoc Holm-Sidak method, with alpha=5%. Each row was analyzed individually, without assuming a consistent SD. Error bars represent SEM.

B. MC PAFR is necessary for PAFR-mediated immunosuppression

Evidence that MCs were necessary for PAFR-mediated immune suppression, led to the hypothesis that the MC PAFR is necessary to 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 sensitization to DNFB and subsequent ear challenge. While WT MC transplantation rescued the immunosuppressive ability of CPAF as measured by CHS, transplantation of PAFR KO MCs failed to rescue this phenotype (Figure 22). Additionally, if WT MCs were transplanted into Wsh mice lacking PAFR (PAFR KO Wsh), CPAF treatment still exerted a significant immunosuppressive effect (Figure 22). To verify that negative results in PAFR KO MC transplantation were not due to poor transplantation yield, the presence of dermal MCs in these mice were demonstrated by Toluidine Blue staining and MC numbers were found to be in relatively normal numbers (Figure 23). Together, this experimental evidence indicates that the MC PAFR is both necessary and sufficient to mediate systemic immunosuppression by PAF.

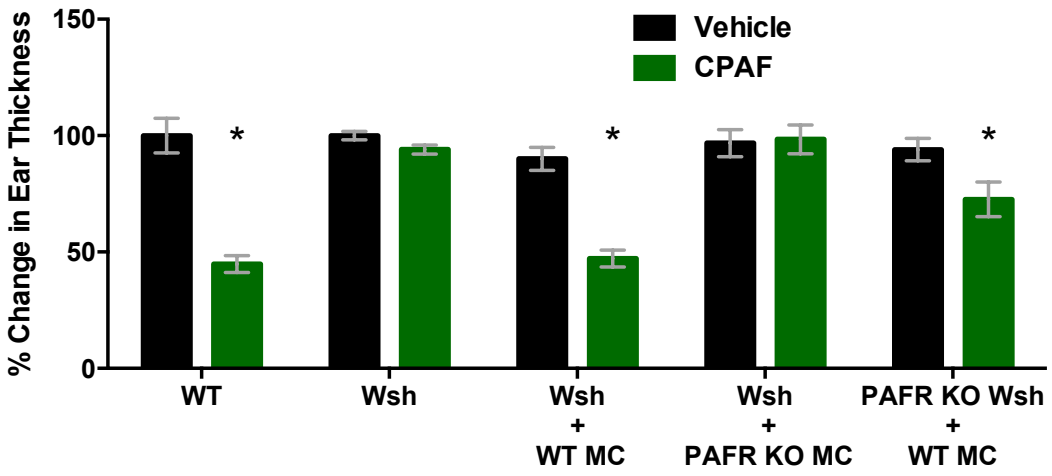


Figure 22. MC PAFR is necessary and sufficient for PAFR-mediated systemic immune suppression.
Groups of 8-9 WT, Wsh, Wsh mice reconstituted with WT, or PAF-R KO MC; or PAFR KO Wsh mice reconstituted with WT MC, were treated with CPAF or vehicle 5 days prior to DNFB sensitization Mice were challenged with DNFB on Day 9 post-challenge and ear thickness was measured 24 hours later. * Denotes statistically significant differences ($p<0.05$) in ear thickness measurements normalized to vehicle treated mice. Statistical significance determined using two-way ANOVA and the post-hoc Holm-Sidak method, with alpha=5%. Each row was analyzed individually, without assuming a consistent SD. Error bars represent SEM. Experiments representative of two separate experiments with 8-9 mice per group in each experiment.

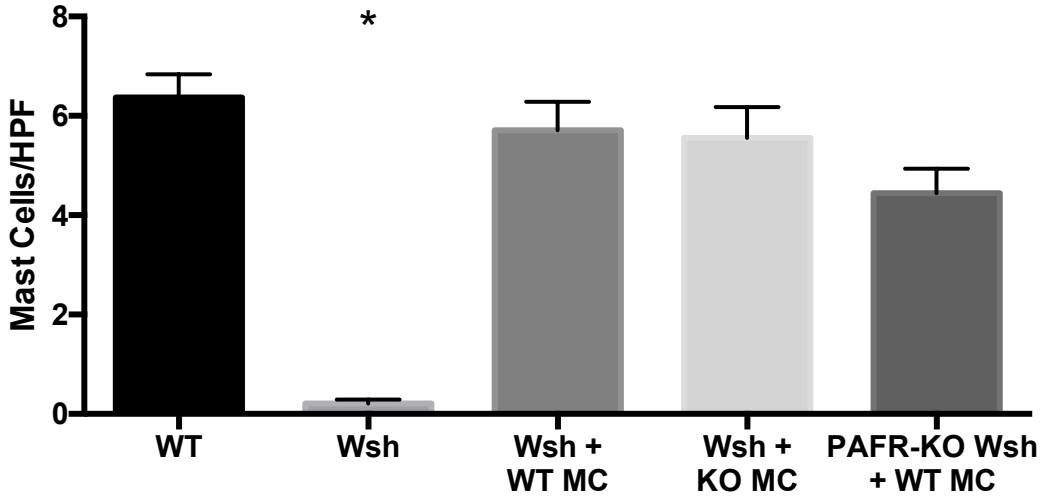


Figure 23. MC counts in dorsal skin slides stained with toluidine blue.
Dorsal skin from mice were formalin fixed before storage in ethanol. Sectioned slides were stained for MCs by acidified toluidine blue. MC numbers were quantified by counting ten high power fields (HPF, 600X). Data depicted is the mean of 4 mice ± SEM. * Denotes statistically significant differences ($p<0.05$) compared to WT. Statistical significance determined using one-way ANOVA and the post-hoc Bonferroni method for multiple corrections, with alpha=5%. Error bars represent SEM.

C. MC-derived histamine is necessary for PAF to induce systemic immunosuppression

In 1998, Finlay-Jones and colleagues published a paper demonstrating that MC deficient mice were not sensitive to immunosuppression by UVB [68]. Furthermore, they showed that subcutaneous injection of histamine could elicit similar decrease in CHS response as UVB in WT mice. These data led to the hypothesis that MC-derived histamine may be one of the mediators involved in the immunosuppressive effects of PAF. To test this hypothesis, WT, PAFR KO, or Wsh mice were treated with vehicle, CPAF, or histamine before sensitization to DNFB and subsequent ear challenge to DNFB for CHS experiments. Unlike CPAF, which has no effect on ear swelling to DNFB challenge in Wsh mice and PAFR KO mice, histamine attenuated ear swelling on WT, PAFR KO and Wsh mice (Figure 24).

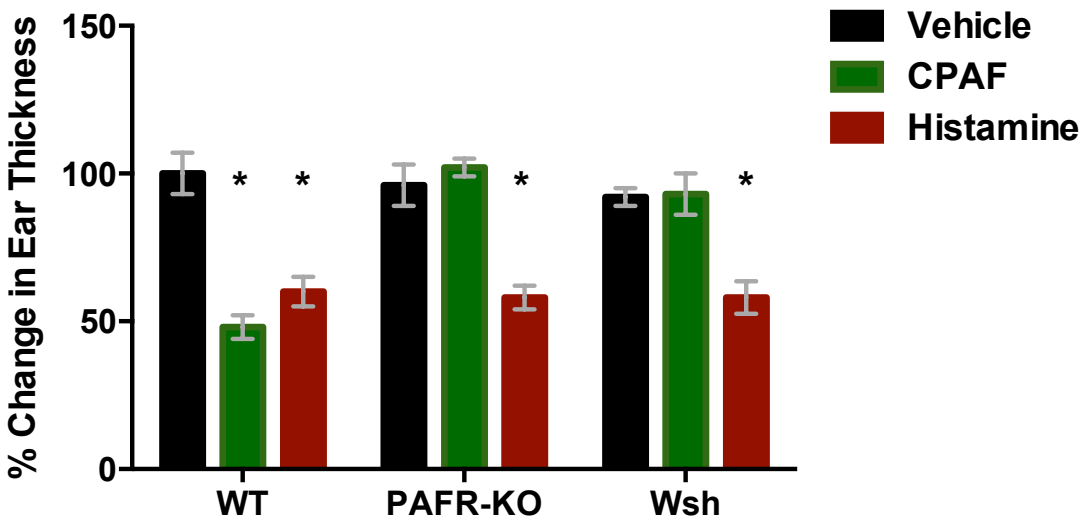


Figure 24. MC deficient mice are insensitive to CPAF- but sensitive to histamine-induced inhibition of CHS reactions.
Groups of 8-12 WT, PAF-R KO or Wsh mice were treated with histamine (200 µg sc) , CPAF (250ng ip), or vehicle 5 days prior to DNFB sensitization. Mice were challenged with DNFB on Day 9 post-challenge and ear thickness was measured 24 hours later. * Denotes statistically significant differences ($p<0.05$) in ear thickness measurements normalized to vehicle treated mice. Statistical significance determined using two-way ANOVA and the post-hoc Holm-Sidak method, with alpha=5%. Each row was analyzed individually, without assuming a consistent SD. Error bars represent SEM.

Subsequently, this evidence led to the hypothesis that histamine is necessary for the immunosuppressive effects of PAF. To test this hypothesis, histidine decarboxylase KO (HDC KO) mice, which lack the enzyme responsible for histamine biosynthesis, were used. Thus, WT and HDC KO mice were treated with vehicle, CPAF or histamine before DNFB sensitization and subsequent CHS challenge. HDC KO mice were found to be sensitive to immunosuppression by histamine, but insensitive to CPAF-induced immunosuppression (Figure 25). This evidence suggests that histamine might be inducing systemic immunosuppression down-stream of PAFR activation.

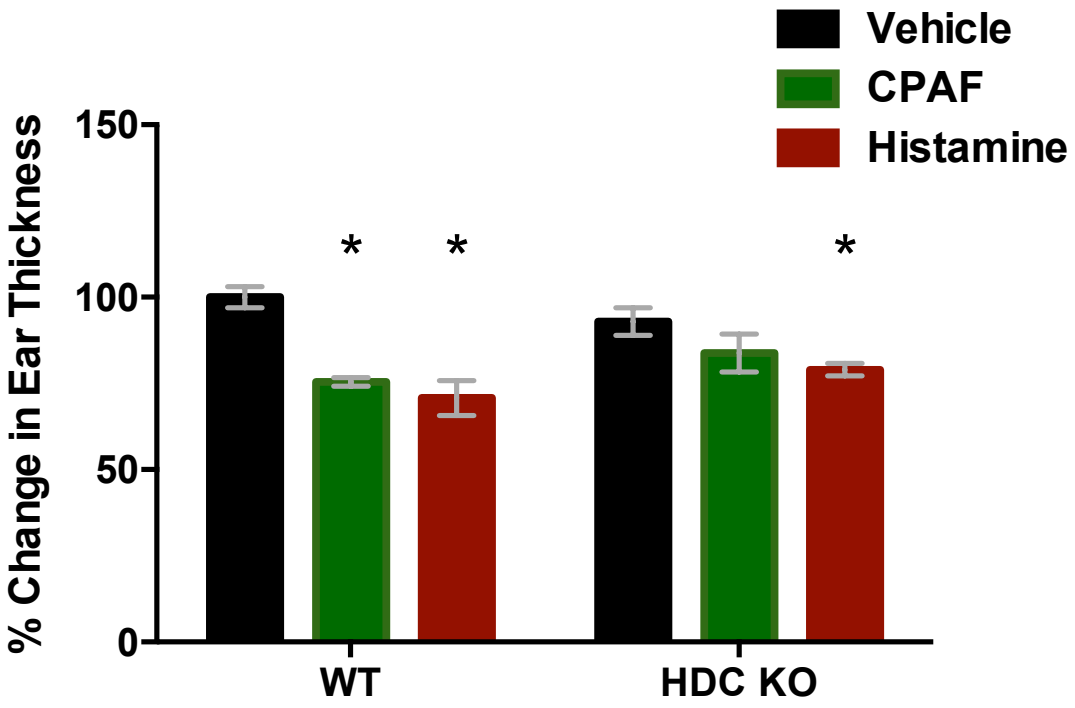


Figure 25. HDC KO mice are insensitive to CPAF- but sensitive to histamine-induced inhibition of CHS reactions.
Groups of 10-15 WT, or HDC KO mice were treated with histamine, CPAF, or vehicle 5 days prior to DNFB sensitization. Mice were challenged with DNFB on Day 9 post-challenge and ear thickness was measured 24 hours later. * Denotes statistically significant differences ($p<0.05$) in ear thickness measurements normalized to vehicle treated mice. Statistical significance determined using two-way ANOVA and the post-hoc Holm-Sidak method, with alpha=5%. Each row was analyzed individually, without assuming a consistent SD. Error bars represent SEM. Figure is representative of three pooled experiments.

MCs are the primary, although not the only, source of histamine. Circulating basophils are also a source of this bioactive amine [145]. As such, to test the hypothesis that PAFR activation can induce MCs to release histamine and that MC-derived histamine is necessary for the immunosuppressive effects of PAF, BMMCs were first treated with CPAF in vitro. Using qRT-PCR, mRNA transcript for *Hdc* was found to be upregulated after incubation with CPAF compared to vehicle treatment (Figure 26A). Additionally, western blotting demonstrated that HDC protein production is increased as well following CPAF treatment (Figure 26B and C). Lastly, CPAF-treated BMMCs were found to have greater concentrations of histamine release, compared to vehicle, as measured by ELISA (Figure 26D). These findings indicate that MCs are capable of releasing histamine following PAFR activation.

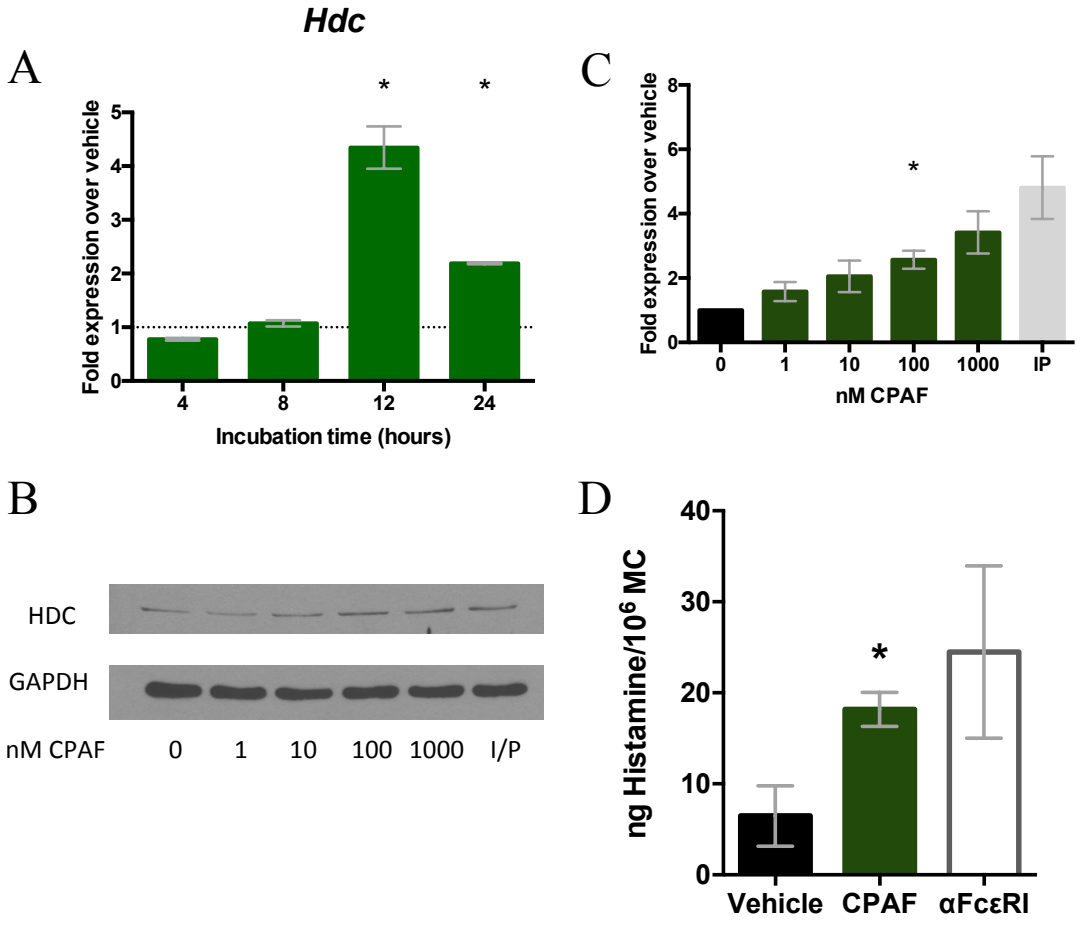


Figure 26. MC PAFR activation increases HDC mRNA and protein expression. **A.** BMMCs were incubated with CPAF (100 nM) for different time points. *Hdc* gene expression relative to vehicle (1-fold) was measured by qRT-PCR using the $2^{-\Delta\Delta CT}$ method and endogenous *Gapdh* internal control. Each time point is representative of three experiments. **B.** BMMCs were treated for 24hrs with increasing doses of CPAF or ionophore/PMA (I/P). Protein fractions of cell lysates were run on an SDS-PAGE gel and proteins were transferred to a nitrocellulose membrane. Membranes were blotted for HDC and GAPDH. Figure is representative of three experiments. **C.** Densitometry analysis of HDC western blots depict average fold expression of three experiments of HDC relative to vehicle (1-fold) using GAPDH as loading control. **D.** Histamine EIA shows average histamine release from three experiments from BMMCs incubated with vehicle, CPAF (100nM) or αFcεRI Ab (3µg/mL, positive control) for one hour. Error bars represent SEM. * Denotes statistically significant differences ($p<0.05$).

Thus far the evidence suggests that histamine signaling and MC PAFR activation are necessary for the immunosuppressive effects of PAF. While, MC PAFR activation induces the release of histamine, MCs are not the only secretors of histamine. These data led to the hypothesis that MC-derived histamine is necessary for PAF-induced immunosuppression. To test this hypothesis MCs were derived from HDC KO mouse BM. HDC KO or WT BMMCs, were then transplanted into Wsh mice. Subsequently, these mice and WT controls were treated with vehicle, CPAF, or histamine, 5 days prior to sensitization to DNFB for CHS experiment. The results of these experiments demonstrated that CPAF and histamine attenuated ear swelling due to antigen challenge in all of the mice (Figure 27A). Surprisingly, these experiments suggest that MC HDC seems to be dispensable for the immunosuppressive effects of CPAF. Since it is known that many cell types express the PAFR, there are likely redundant mechanisms in this pathway. In particular, basophils, which express both PAFRs and produce histamine, could serve this redundant role. For this reason, the use of a PAFR KO Wsh mouse, which not only lacks MCs, but also lacks PAFR expression, could be useful in isolating the role of MC HDC in PAFR-mediated immunosuppression. This mouse is useful in transplantation, because transplanting PAFR-expressing MCs isolates PAFR expression to only one cell type. Using a similar approach, WT or HDC KO BMMCs were transplanted into the dorsal skin of PAFR KO Wsh mice. Transplanted mice and WT control mice were treated with vehicle, CPAF or histamine, prior to DNFB sensitization. After DNFB challenge, histamine treatment blocked ear-swelling responses in all groups, however, CPAF treatment failed to suppress the DNFB responses in PAFR KO Wsh mice transplanted with HDC KO MCs (Figure 27B).

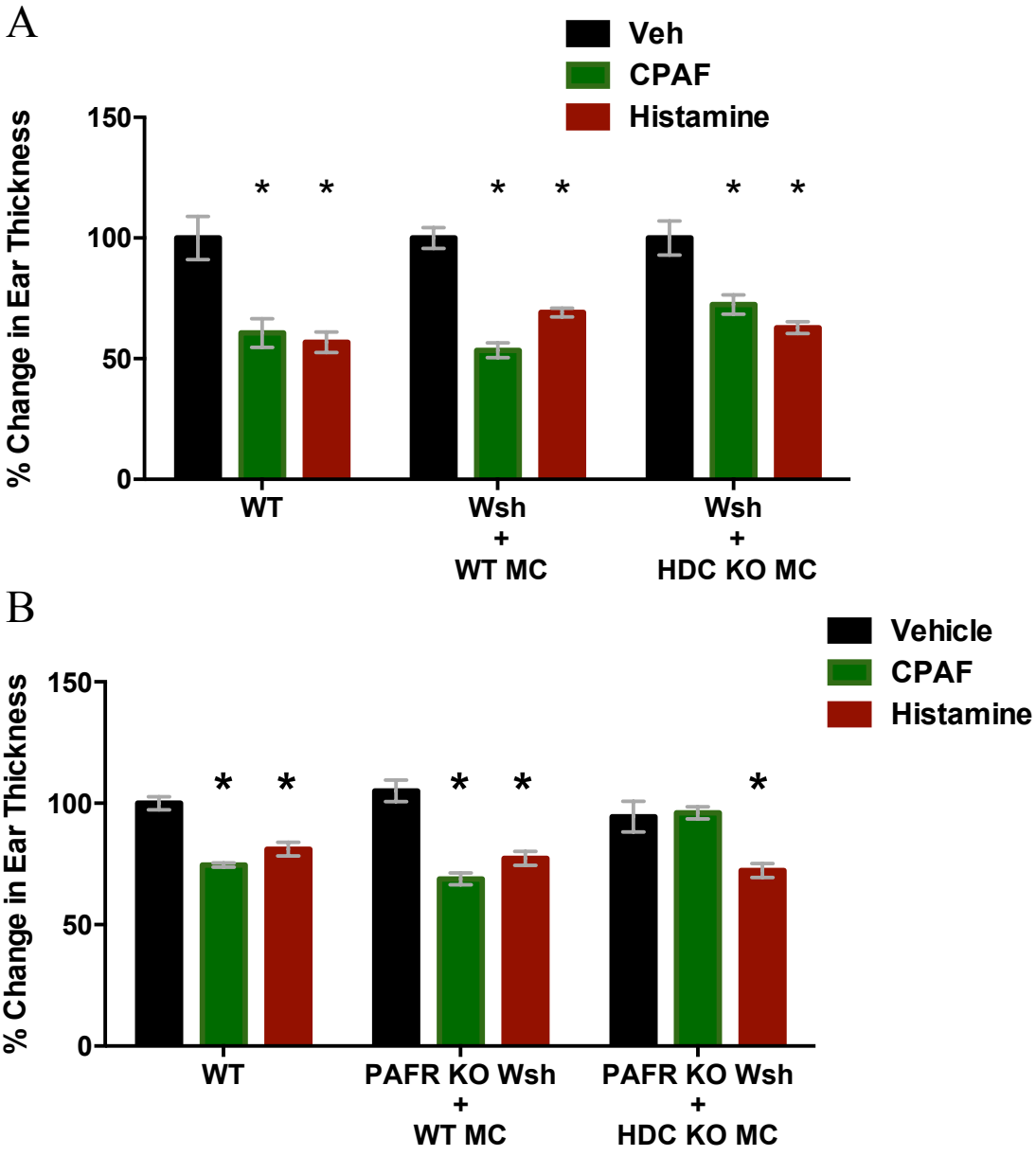


Figure 27. MC-derived histamine is necessary for PAFR inhibition of CHS response.

WT or HDC KO MCs were transplanted into the dorsal skin of Wsh (**A**) or PAFR KO/ Wsh (**B**) mice. Groups of 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. CPAF does not confer immunosuppression in PAFR KO Wsh mice reconstituted with HDC KO MCs. Error bars represent SEM. * Denotes statistically significant differences ($p<0.05$) in ear thickness measurements normalized to vehicle treated mice. Statistical significance determined using two-way ANOVA and the post-hoc Holm-Sidak method, with alpha=5%. Each row was analyzed individually, without assuming a consistent SD. Experiments representative of two different experiments with n=5-7 mice per group in each experiment.

These data suggest that MC-derived histamine plays a role in PAFR-mediated immunosuppression. While not necessary, MC-derived histamine may be sufficient to induce systemic immunosuppression down-stream of PAFR activation.

D. MC COX-2 is necessary for PAF-induced immunosuppression

Previous work has demonstrated the importance of prostaglandins for UV light to suppress the immune system. In particular, Narumiya and colleagues recently showed that the prostaglandin receptor EP4 mediates UV-induced systemic immunosuppression [131]. Our group has previously shown using a CHS assay that immunosuppression induced by UVB, cigarette smoke and CPAF can all be blocked by COX-2 inhibitors [24, 25, 135]. In a similar fashion, WT mice were also treated with COX-2 inhibitor SC-236 and vehicle, CPAF or histamine, 5 days prior to sensitization to DNFB. After DNFB challenge, ear measurements demonstrated that COX-2 inhibitors can block the immunosuppressive effects of CPAF and histamine (Figure 28).

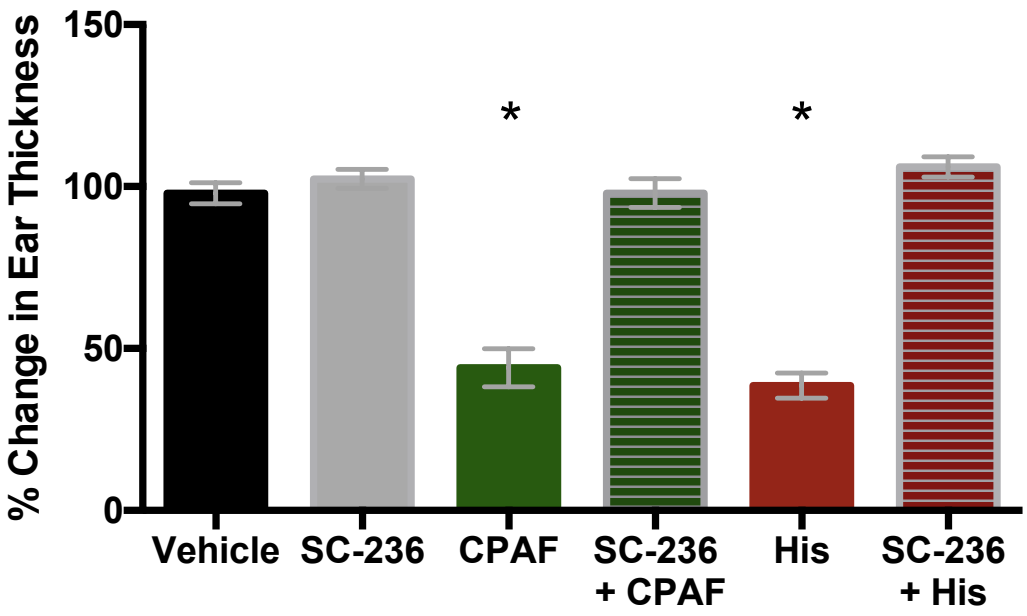


Figure 28. COX-2 inhibitor SC-236 blocks CPAF- and histamine-induced inhibition of CHS responses.
Groups of 6-8 mice along with WT were treated with vehicle or SC-236 (200 ng), and vehicle, CPAF i.p. or histamine s.c. 5 days before sensitization to DNFB for CHS assay. Error bars represent SEM. * Denotes statistically significant differences ($p<0.05$) in ear thickness measurements normalized to vehicle treated mice. Statistical significance determined using one-way ANOVA and the post-hoc Dunnett's method, with alpha=5%. Each row was analyzed individually, without assuming a consistent SD. Experiments representative of two different experiments with n=4-5 mice per group in the second experiment.

These data suggest that COX-2 is involved in mediating the suppressive response of PAF, but possibly down-stream of histamine. To test the hypothesis that MC PAFR activation could induce prostaglandin release, BMMCs were first treated with CPAF. qPCR and western blotting, revealed that COX-2 mRNA and protein levels are upregulated in BMMCs treated with CPAF (Figure 29A,B and C). Additionally, as shown in Figure 29D, treating BMMCs with CPAF resulted in PGE_2 release in BMMCs. These findings demonstrate that MC PAFR activation induces COX-2 expression as well as prostaglandin release.

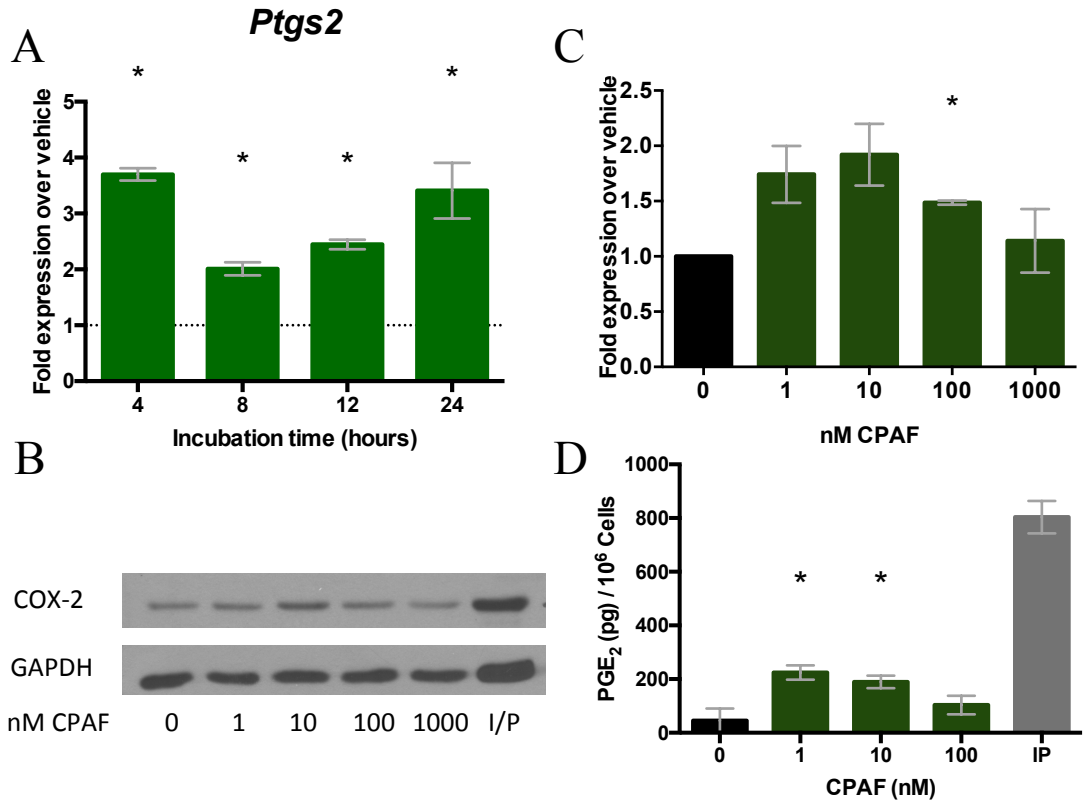


Figure 29. MC PAFR activation results in COX-2 mRNA and protein expression, and PGE_2 production.

A. BMMCs were incubated with CPAF (100 nM) for different time points. *Ptgs2* gene expression relative to vehicle (1-fold) was measured by qRT-PCR using the $2^{-\Delta\Delta CT}$ method and endogenous *Gapdh* internal control. Figure is representative of three separate experiments. **B**. BMMCs were treated for 24hrs with increasing doses of CPAF or ionophore/PMA (I/P). Protein fractions of cell lysates were run on an SDS-PAGE gel and proteins were transferred to a nitrocellulose membrane. Membranes were blotted for COX-2 and GAPDH. Figure is representative of three separate experiments. **C**. Densitometry analysis of COX-2 western blots depict average fold expression of three experiments of HDC relative to vehicle (1-fold) using GAPDH as loading control. **D.** PGE_2 EIA shows PGE_2 release from BMMCs incubated with vehicle, ionophore/PMA (I/P), or increasing concentrations of CPAF at eight hours. Figure is representative of three separate experiments. Error bars represent SEM. * Denotes statistically significant differences ($p<0.05$).

Given the findings that PAFR activation can induce COX-2 expression in MCs, thus led to hypothesis that MC COX-2 plays a role in the immunosuppressive mechanism of PAF. In collaboration with Drs. Garret Fitzgerald and Sven-Christian Pawelzik at the University of Pennsylvania, MCs were derived from the BM of *Mcpt5-cre/Ptgs2*$^{flox/flox}$ mice for transplantation into Wsh mice and subsequent CHS experiments [146-148]. Attempts at generating germline COX-2 KO mice on a C57BL/6 background resulted in unhealthy mice that fail to thrive and have reproductive complications [149]. To take a parallel approach, Fitzgerald and Pawelzik have generated COX-2 floxed mice that have a MC-specific driven cre recombinase. MCPT5, mast cell protease 5, is a mast cell specific protease that is expressed in dermal MCs, which are connective tissue type MCs. Thus, BMMCs were cultured from the BM of these mice. BMMCs from *Mcpt5-cre/Ptgs2*$^{flox/flox}$ mice transplanted into Wsh mice will mature and differentiate normally, as has been demonstrated in the literature [69, 147, 148, 150], into dermal mast cells that express MCPT5. Thus, MCPT5 expression will result in the excision of exons VI - VIII from COX-2 resulting in selective loss of COX-2 activity expression. Using this approach BMMCs derived from *Mcpt5-cre/Ptgs2*$^{flox/flox}$ (COX-2 KO) or *Ptgs2*$^{flox/flox}$ (COX-2 WT) mice were transplanted into Wsh mice. These mice and WT controls were treated with vehicle, CPAF, or histamine, 5 days prior to sensitization to DNFB. After DNFB challenge, ear measurements showed that histamine and CPAF reduced ear swelling 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 immunosuppression by CPAF (Figure 30). These findings suggest that MC COX-2 contributes the suppressive effects of PAF on the immune system.

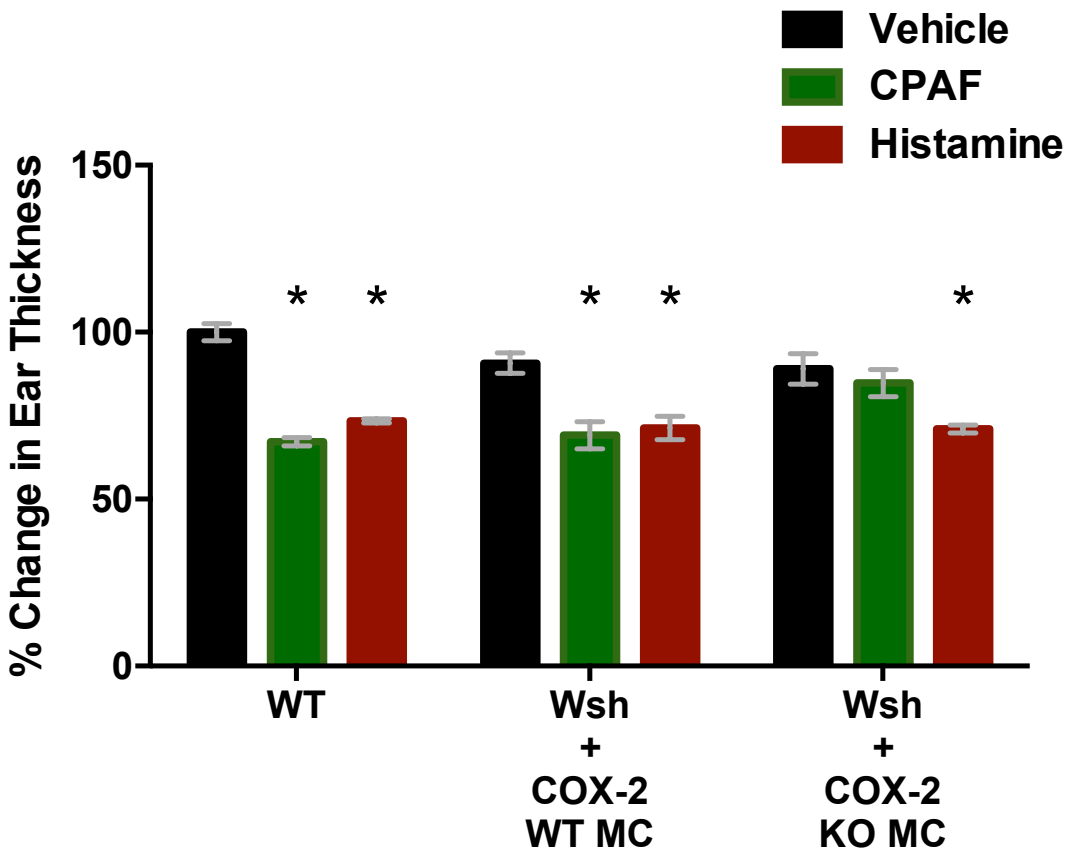


Figure 30. MC COX-2 is necessary for CPAF- but not histamine-induced inhibition of CHS responses.
WT (*Ptgs2*$^{flox/flox}$) or COX-2 KO (*Mcpt5-cre/Ptgs2*$^{flox/flox}$) MCs were transplanted into the dorsal skin of Wsh mice. Groups of 7-10 mice along with WT were treated with vehicle, CPAF i.p. or histamine s.c. 5 days before sensitization to DNFB for CHS assay. CPAF does not confer immunosuppression in Wsh mice reconstituted with COX-2 KO MCs. Error bars represent SEM. * Denotes statistically significant differences ($p<0.05$) in ear thickness measurements normalized to vehicle treated mice. Statistical significance determined using two-way ANOVA and the post-hoc Holm-Sidak method, with alpha=5%. Each row was analyzed individually, without assuming a consistent SD. Experiments representative of two different experiments each containing groups of n=5-10 mice per group in each experiment.

E. The role of TGFβ in PAFR-mediated systemic immunosuppression

Tregs are one of the main cell mediators of tolerance and anti-inflammatory processes and they mediate their effects through various mechanisms including the release of IL-10 and TGFβ [151-153]. While natural Tregs persist following thymic selection, inducible Tregs (iTregs) differentiate from naïve T cells in the presence of TGFβ in their microenvironment [151]. These iTregs play a critical role in attenuating the inflammatory tide. Our group recently published data suggesting that Tregs might be involved in mediating the immunosuppressive effects of PAF [24, 25]. These findings led to the hypothesis that TGFβ may also be involved in the immunosuppressive effects of PAF by virtue of its role in Treg differentiation. To test this hypothesis, WT mice were injected with the TGFβR1 inhibitor LY364947 or vehicle at the time of treatment with vehicle, CPAF or histamine, 5 days prior to sensitization to DNFB. After DNFB ear challenge, ear measurements demonstrated that treating mice with LY364947 blocked the suppressive effects of CPAF and histamine on CHS reactions to DNFB (Figure 31).

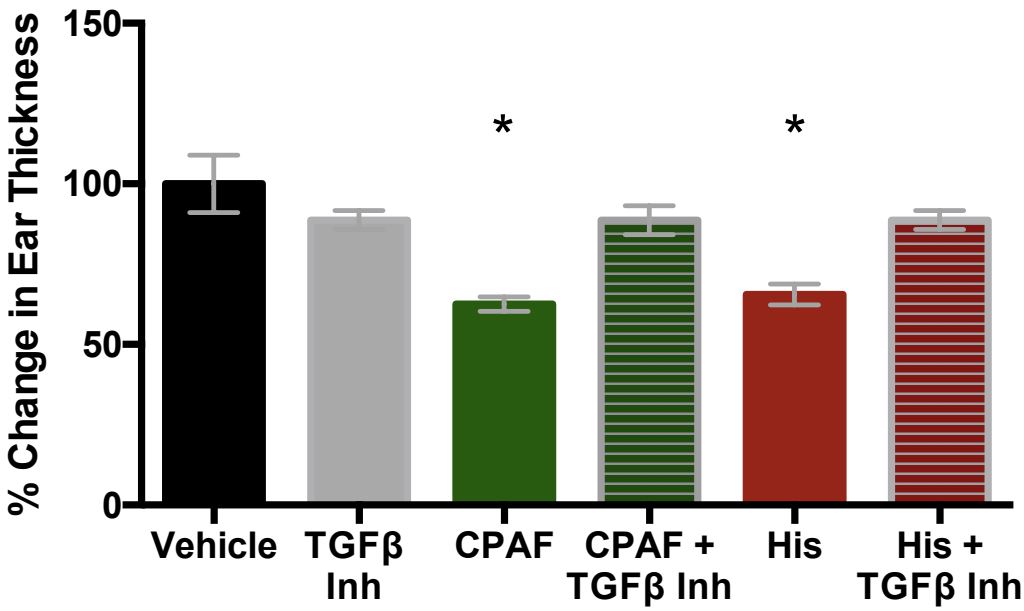
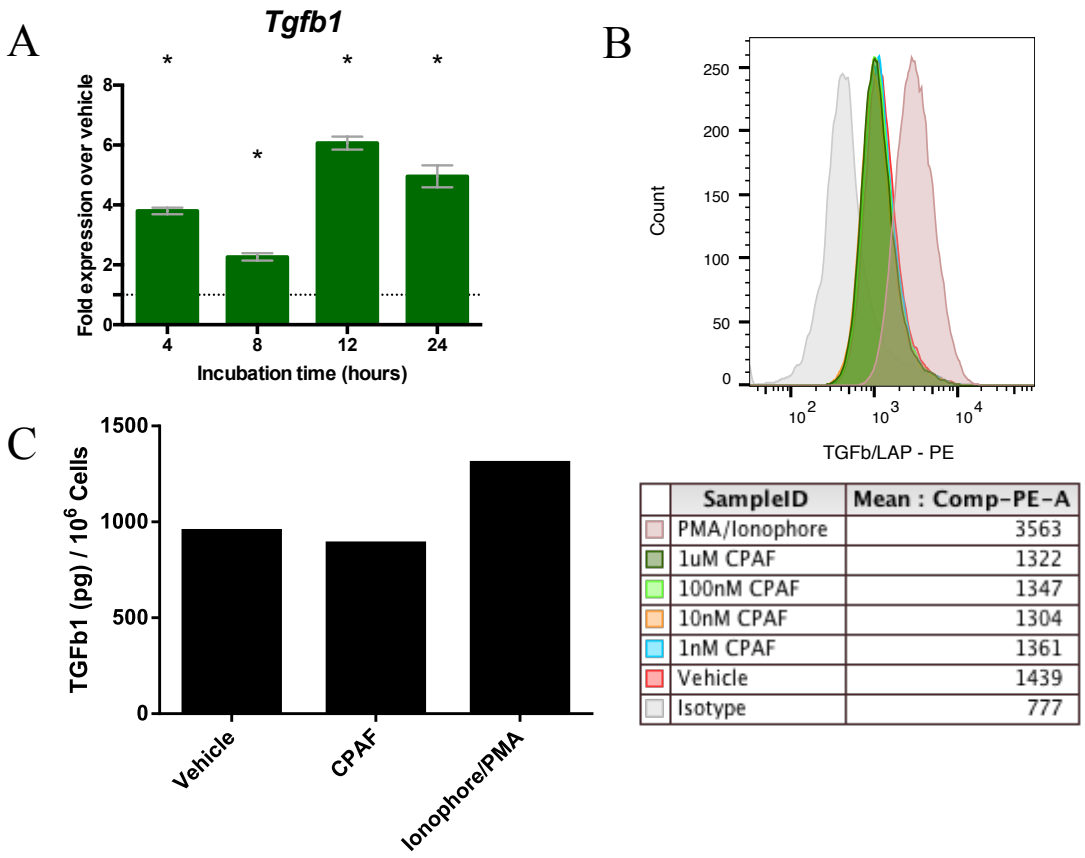


Figure 31. TGFβR1 inhibitor LY364947 blocks for CPAF- and histamine-induced inhibition of CHS responses.
Groups of 5 WT mice were treated with vehicle or TGFβ-R1 inhibitor LY364947 1mg/kg q.o.d. i.p., and CPAF i.p., Histamine s.c., or vehicle 5 days prior to DNFB sensitization. Ears were measured 24hrs after DNFB challenge on Day 9. Error bars represent SEM. * Denotes statistically significant differences ($p<0.05$) in ear thickness measurements normalized to vehicle treated mice. Statistical significance determined using two-way ANOVA and the post-hoc Holm-Sidak method, with alpha=5%. Each row was analyzed individually, without assuming a consistent SD. Figure is representative of two different experiments each with n=5 per group.

These data led to the hypothesis that MC PAFR stimulation might induce TGFβ release. To test this hypothesis, BMMCs were treated with CPAF and qPCR analysis demonstrated that *Tgfb1* mRNA transcript is upregulated after 4 hours (Figure 32A). However, flow cytometry analysis of intracellular staining or ELISA did not provide evidence of increased TGFβ protein expression or increased levels of released TGFβ1 protein (Figure 32B and C). These studies suggest that while TGFβ may be required for PAF to promote the suppression of immune responses, MC PAFR activation does not appear to induce MCs to release TGFβ. It is noteworthy, however, that MCs do readily secrete significant amounts of TGFβ and while release is not induced by PAF, MC migration to lymph nodes could effectively deliver TGFβ to a site of T cell differentiation in a PAFR-independent manner. This scenario where TGFβ exerts its effects down-stream of MC PAFR activation could explain how a TGFβR inhibitor could block CPAF- and histamine-mediated inhibition of CHS reactions. Thus, the MC PAFR could be important in trafficking the MC to the lymph nodes but not for producing the necessary TGFβ.



SampleID	Mean : Comp-PE-A
PMA/Ionophore	3563
1uM CPAF	1322
100nM CPAF	1347
10nM CPAF	1304
1nM CPAF	1361
Vehicle	1439
Isotype	777

Figure 32. CPAF treatment of BMMC results in increased TGFβ1 mRNA but not protein levels.
A. BMMCs were incubated with CPAF (100 nM) for different time points. *Tgfb1* gene expression relative to vehicle (1-fold) was measured by qRT-PCR using the $2^{-\Delta\Delta CT}$ method and endogenous *Gapdh* internal control. Figure is representative of three separate experiments. **B.** BMMCs were treated for 24hrs with increasing doses of CPAF or ionophore/PMA (I/P). Cells were permeabilized with saponin and stained for TGFβ-bound LAP. Figure is representative of three experiments. Mean fluorescence intensities are depicted in the table below. **C.** TGFβ ELISA shows TGFβ1 release from BMMCs incubated with vehicle, ionophore/PMA (I/P), or CPAF for 24 hours. Figure is representative of three experiments. Error bars represent SEM. * Denotes statistically significant differences ($p<0.05$).

F. PAF induces MC migration to draining LNs

Recent work in the field has demonstrated that once MCs are activated by UVB or PAF, MCs subsequently migrate to draining LNs. In particular Ullrich and colleagues have shown that this MC migration may be blocked by AMD3100, a CXCR4 inhibitor, resulting in reduced sensitivity to immunosuppression by CPAF and UVB. Additionally, this group has reported that PGE_2 and UVB treatment upregulates CXCL12, the ligand for CXCR4, in inguinal LNs [73, 74]. Together, these data suggest that MCs migrate to draining LNs following PAFR activation. Still, it has been shown in the literature that CXCR4 is regulated by prostaglandins including PGE_2, although there is some debate as to whether prostaglandins promote or inhibit migration [38, 154-158]. These findings led to the hypothesis that PAF-induced MC prostaglandin release might be the down-stream effectors of the MC PAFR by regulating MC CXCR4 expression. To show that 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. Bilateral inguinal LNs were harvested 24 hours later. One LN was formalin fixed, sectioned and stained for MCs with toluidine blue. The other LN was passed through a 70μm filter to render a single cell suspension that was stained and subjected to flow cytometry analysis. Histology revealed the presence of significant numbers of MCs in LNs from mice treated with CPAF (Figure 33). Interestingly, these mast cell populations were absent in mice treated with both CPAF and SC-236, suggesting that COX-2 inhibitors could block the PAFR-mediated migration of MCs to LNs. Flow cytometry analysis revealed similar findings (Figure 34).

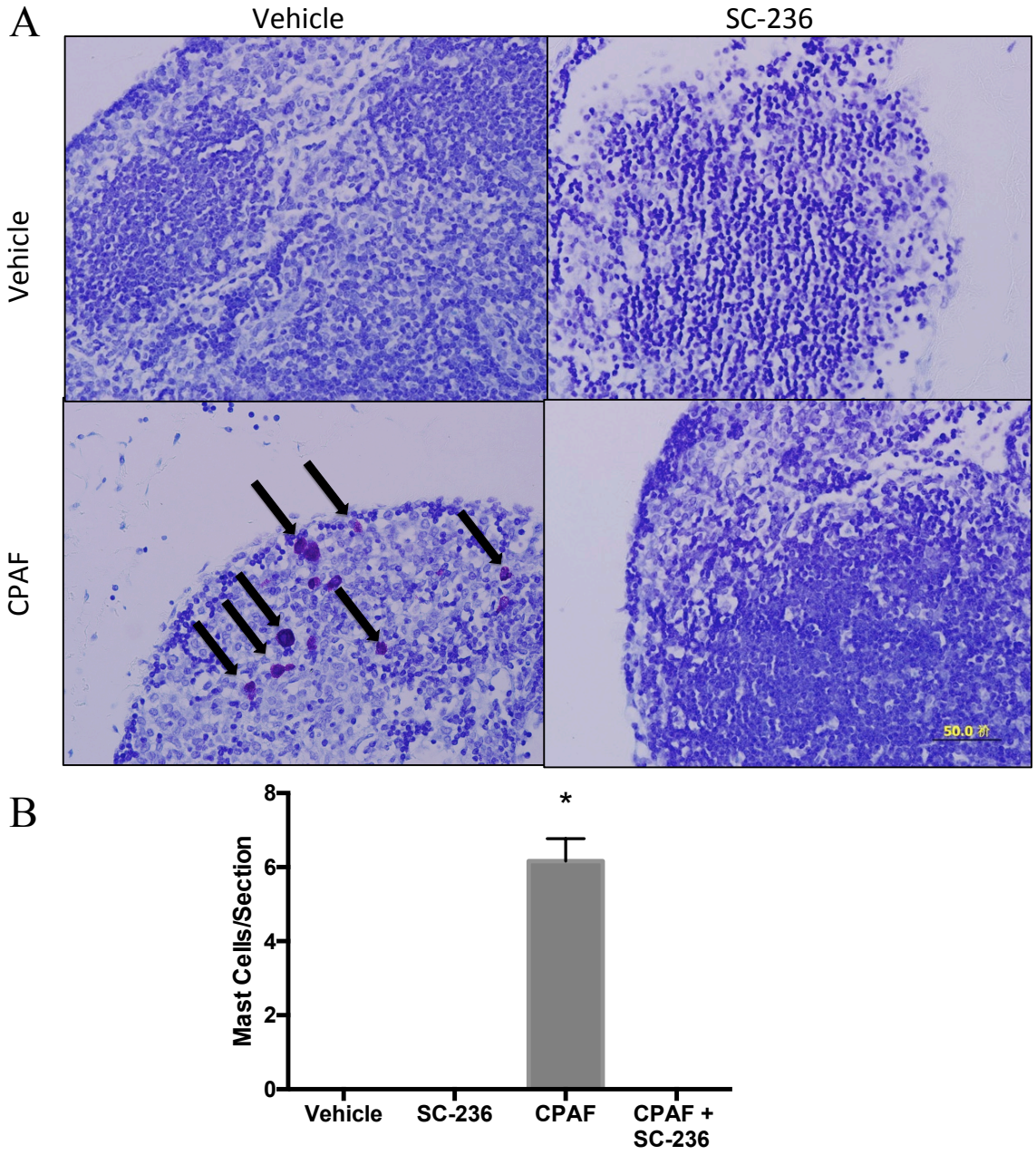


Figure 33. Histological analysis of PAF-induced MC migration to LNs blocked by COX-2 inhibitors.

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 50µm. **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 ± SEM of three slides from 1-2 mice per group. Error bars represent SEM. * Denotes statistically significant differences ($p<0.05$).

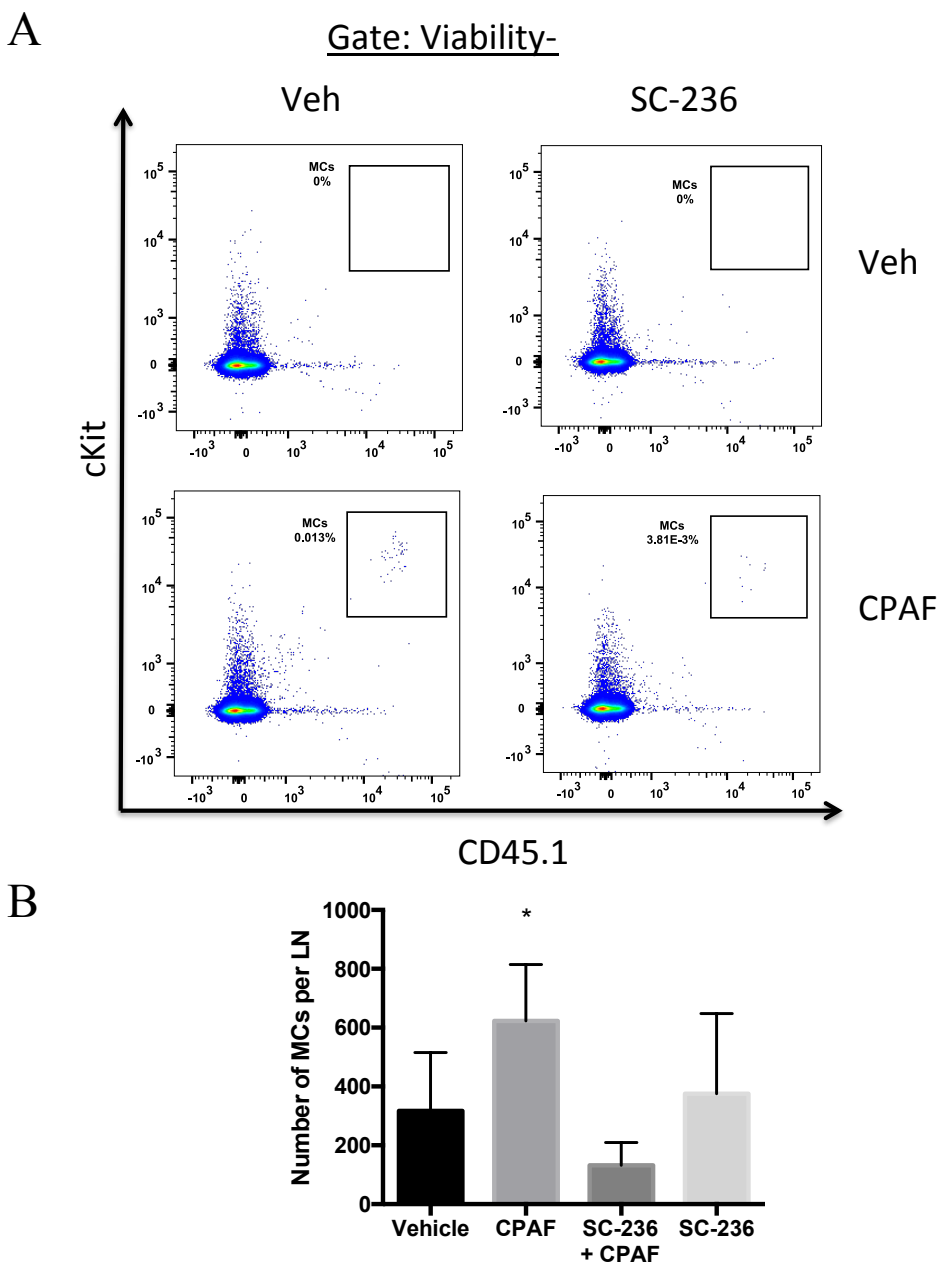


Figure 34. Flow cytometry analysis of PAF-induced MC migration to LNs blocked by COX-2 inhibitors.

A. CD45.1 BOYJ BMMCs (10^6) were transplanted into the dorsal skin of Wsh mice. Groups of 3 mice were then treated with vehicle or CPAF, and vehicle or SC-236. Inguinal LNs were harvested 24hrs later and passed through a 70µm filter to make a single cell suspension. Cells were stained and analyzed by flow cytometry. Figure depicts representative dot plots of cells that are Live ckit+ CD45.1+ CD4-. **B.** Total number of MCs per LN were calculated. Mean MC numbers of 4 separate experiments are shown. Error bars represent SEM. * Denotes statistically significant differences ($p<0.05$).

Subsequent experiments demonstrated that CPAF treated MCs upregulate CXCR4 transcript after 12 hours (Figure 35A). Flow cytometry analysis also demonstrated that CXCR4 is upregulated in the surface of MCs treated with CPAF as compared to vehicle treated cells. Interestingly, however, incubation of these cells with COX-2 inhibitor SC-236 could block this increase in surface CXCR4 expression (Figure 35B). Additionally, incubation of MCs with EP4 agonist CAY10598 resulted in increases surface expression of CXCR4 in MCs (Figure 35C). These data suggest that MC CXCR4 expression may be regulated by prostaglandins acting in an autocrine or paracrine fashion by activating the MC EP4 receptor. In other words, it seems that the machinery needed for PAFR activated MCs to migrate to LNs is regulated by prostaglandins.

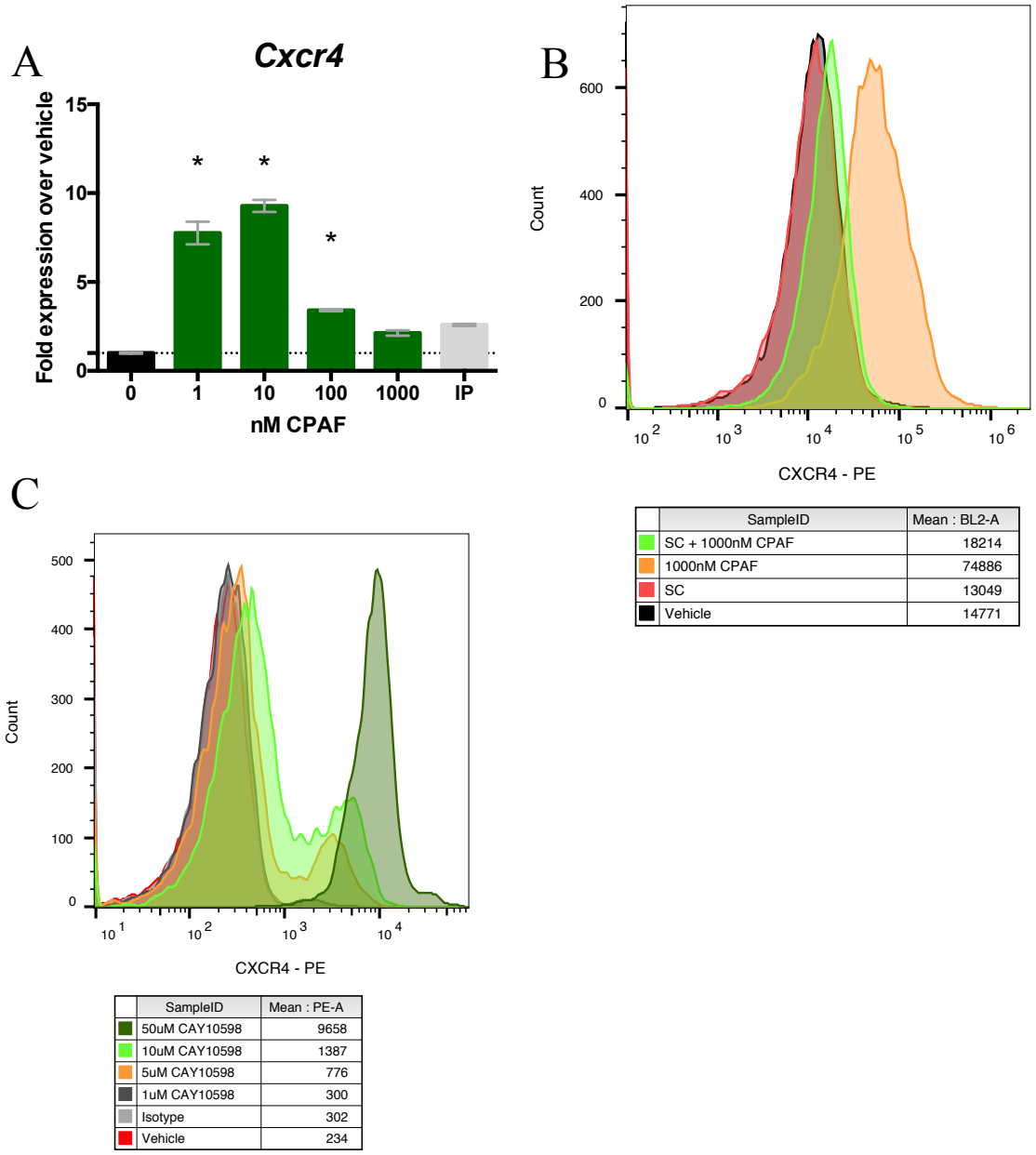


Figure 35. CPAF upregulation of CXCR4 is blocked by COX-2 inhibitor SC-236, while activation of EP4 is sufficient to increase MC surface CXCR4 expression. **A.** BMMCs were incubated with increasing concentrations of CPAF for 12hrs. *Cxcr4* gene expression relative to vehicle (1-fold) was measured by qRT-PCR using the $2^{-\Delta\Delta CT}$ method and endogenous *Gapdh* internal control. Figure is representative of three experiments. **B.** BMMCs were treated for 24hrs with vehicle, CPAF (**B**), EP4 agonist CAY10598 (**C**) or ionophore/PMA (I/P), and vehicle or SC-236 (500 nM). Cells were stained for surface CXCR4 expression with a conjugated antibody. Figures are representative of three experiments. Error bars represent SEM. * Denotes statistically significant differences ($p<0.05$).

Ongoing experiments are aimed at investigating the functional role of PAF-induced MC histamine release in MC migration to LNs. It has been previously found that histamine can directly upregulate COX-2 expression in cells [159] and that UV-induced production of prostaglandins in the skin can be mediated by histamine [160]. Additionally, it has been found that secretory granules released from MCs can travel down lymphatic vessels to affect distant LN microenvironments [81], and that PGE_2 can upregulate CXCL12, the chemokine ligand necessary for CXCR4-mediated migration, in inguinal LNs [73]. These findings led to the hypothesis that histamine released from MCs could upregulate to COX-2 in LNs to provide the prostaglandins necessary for CXCL12 release and thus provide a chemokine gradient for CXCR4-mediated MC migration to draining LNs. To test this hypothesis, preliminary experiments consisted of injecting histamine subcutaneously, or PBS in the dorsal skin of WT mice. After one hour, draining LNs were harvested and homogenized. Western blotting from the LN lysates revealed increased COX-2 protein expression in draining LNs (Figure 36). These data support the hypothesis that histamine may play a role in setting up the chemokine gradient for MC migration to draining LNs. Yet, further work is needed to fully delineate the mechanism of PAF-induced MC migration to draining LNs in the context of subsequent systemic immunosuppression.

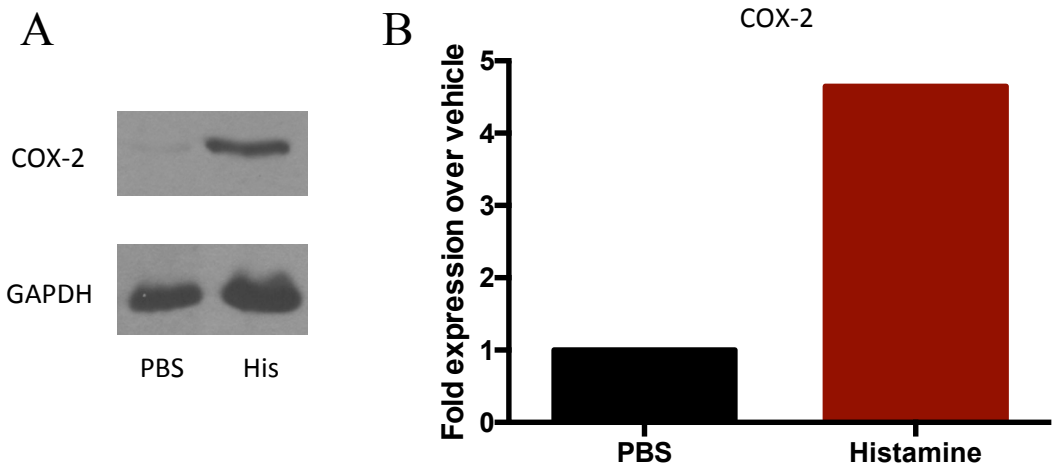


Figure 36. Subcutaneous histamine increases COX-2 expression in skin DLNs. **A.** Histamine (200 µg in 100µL s.c.) or PBS (100µL) was injected into the dorsal skin of WT mice. Inguinal LNs were harvested one hour later. Whole LNs were homogenized by mechanical syringe methods while on ice. Lysates were separated by polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and blotted for COX-2 and GAPDH by western blotting techniques. **B.** Densitometry analysis of COX-2 western blots depict average fold expression of three experiments of HDC relative to vehicle (1-fold) using GAPDH as loading control. Error bars represent SEM. * Denotes statistically significant differences ($p<0.05$).

G. IL-33 signaling plays a role in PAF-induced systemic immunosuppression

Recently, UVB irradiation, as well as systemic CPAF treatment, has been shown to increase the expression of alarmin IL-33 in the skin [161]. Additionally, treatment with high dose IL-33 has been shown to reduce the Th1 response in cell mediated hypersensitivity [161]. This evidence led to the hypothesis that IL-33 may be necessary for PAF to suppress the immune system following UVB irradiation. To test this hypothesis, mice lacking the IL-33 receptor, ST2 KO mice, were tested for the sensitivity to immunosuppression by UVB or CPAF. Thus, ST2 KO mice were treated with vehicle, UVB, CPAF, or histamine, five days prior to sensitization with DNFB. A CHS assay revealed that UVB and histamine showed a robust decrease in ear swelling following challenge to DNFB (Figure 37). CPAF, however, demonstrated some decrease in ear swelling, but still lesser in magnitude than UVB irradiation (Figure 37). To follow up on this experiment, WT and ST2 KO mice were treated with vehicle, CPAF, or histamine prior to elicitation of CHS responses. ST2 KO mice were found to be less sensitive to the suppressive effects of CPAF on CHS responses (Figure 38). Similar results were found using IL-33 KO mice.

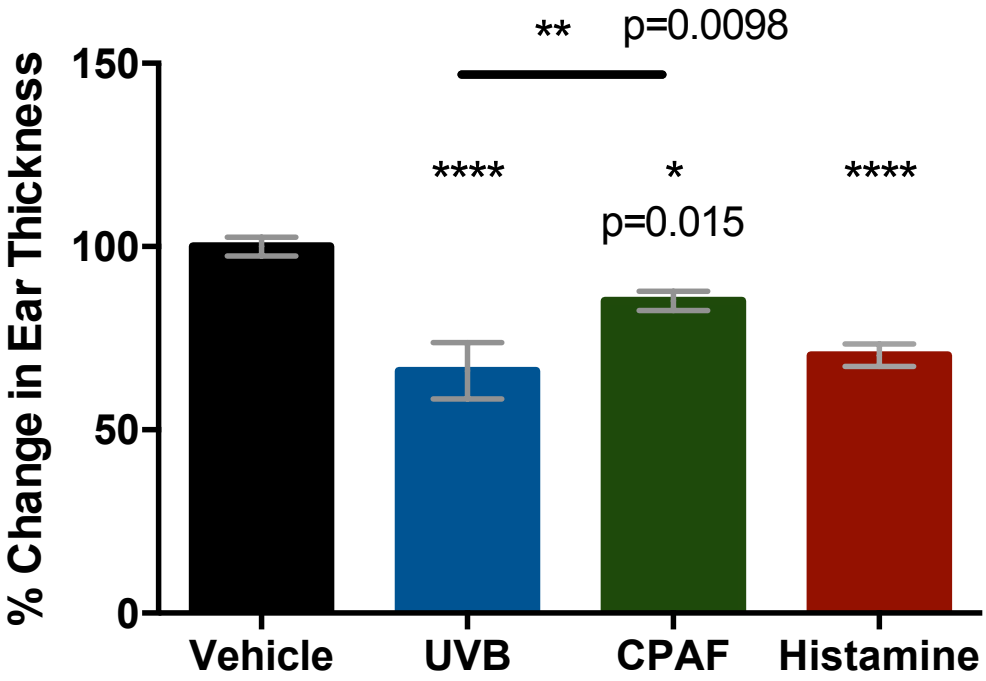


Figure 37. ST2 KO mice are sensitive to UVB- and histamine-induced but have decreased sensitivity to CPAF-induced inhibition of CHS reactions.
Groups of 5-10 ST2 KO mice were treated with vehicle, UVB, CPAF, or histamine 5 days prior to DNFB sensitization. Mice were challenged with DNFB on Day 9 post-challenge and ear thickness was measured 24 hours later. * Denotes statistically significant differences ($p<0.05$) in ear thickness measurements normalized to vehicle treated mice. Statistical significance determined using one-way ANOVA and the post-hoc Sidak method, with alpha=5%. Each row was analyzed individually, without assuming a consistent SD. Error bars represent SEM. Figure is representative of one experiment.

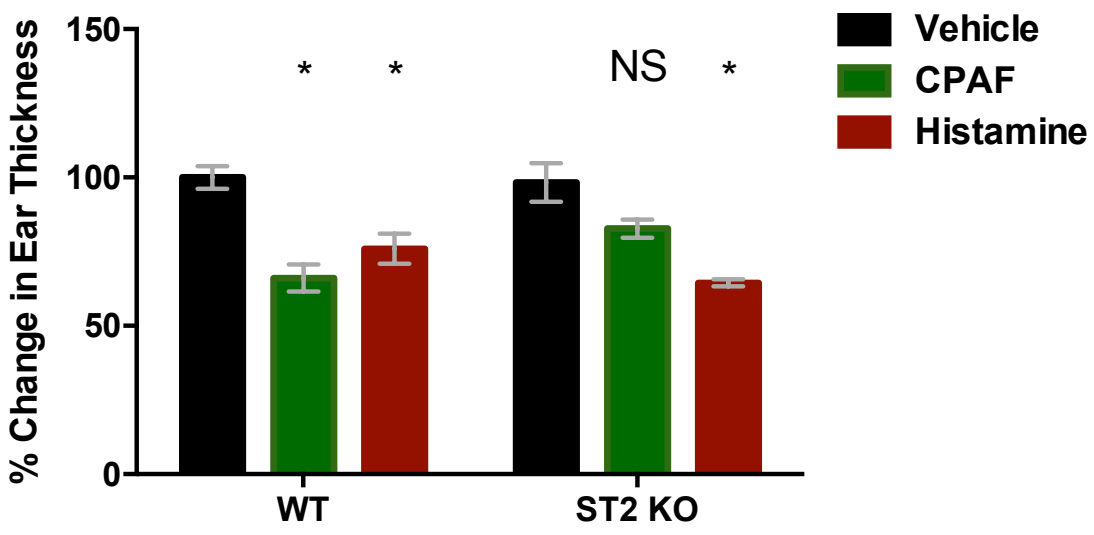


Figure 38. CPAF does not suppress CHS response in ST2 KO mice.
Groups of 5 WT or ST2 KO mice were treated with vehicle, CPAF, or histamine 5 days prior to DNFB sensitization. Mice were challenged with DNFB on Day 9 post-challenge and ear thickness was measured 24 hours later. * Denotes statistically significant differences ($p<0.05$) in ear thickness measurements normalized to vehicle treated mice. Statistical significance determined using two-way ANOVA and the post-hoc Holm-Sidak method, with alpha=5%. Each row was analyzed individually, without assuming a consistent SD. Error bars represent SEM. Figure is representative of three experiments with n=5 mice per group per experiment.

There has been a large body of work demonstrating that MCs mediate inflammatory mechanisms following stimulation by IL-33. Particularly in arthritis models, MCs have been shown to be involved in a perpetual IL-33/TNFα cycle [162, 163]. These findings led the hypothesis that IL-33 signaling may be involved in the mechanism by which MCs mediate the immunosuppressive effects of PAF. To test this hypothesis, first WT BMMCs were tested for expression the IL-33 receptor by flow cytometry, and were found to express ST2 (Figure 39A). CPAF and PGE_2 were also found to upregulate ST2 expression in MCs by qPCR (Figure 39B and C). Then, BMMCs from WT, IL-33 KO or ST2 KO mice were transplanted into the dorsal skin of Wsh mice. These mice as well as WT controls were treated with vehicle, CPAF, or histamine, 5 days prior to sensitization to DNFB and subsequent ear challenge to DNFB. Wsh mice transplanted with WT and IL-33 KO MCs were found to be sensitive to the immunosuppressive effects of CPAF on ear challenge responses (Figure 40). In mice transplanted with ST2 KO MCs, however, CPAF did not demonstrate significant reduction in response to DNFB. Similar results were observed in PAFR KO Wsh mice transplanted with WT or ST2 KO MCs (Figure 41). This evidence suggests that while IL-33 may play a role in mediating PAF-induced immunosuppression, MC activation by IL-33, but not its release by MCs, may be involved.

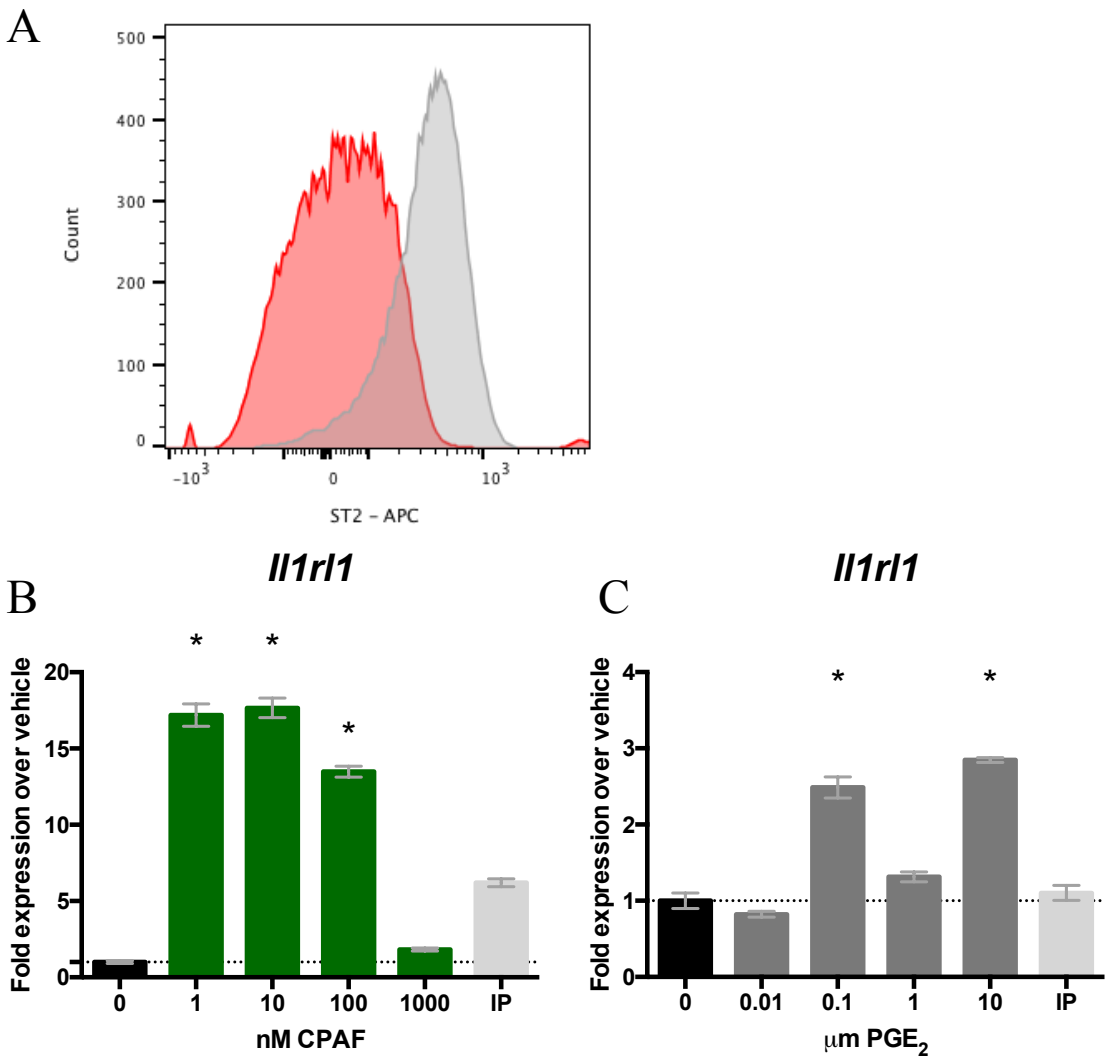


Figure 39. MCs express ST2 which is upregulated by CPAF and PGE_2.
A. BMMCs were unstained (red) or stained with APC-conjugated ST2 antibody (grey) and analyzed by flow cytometry. Figure is representative of three experiments. **B and C.** BMMCs were incubated with increasing concentrations of CPAF (12 hrs, **B**), PGE_2 (4 hrs, **C**) or IP. *Il1rl1* gene expression relative to vehicle (1-fold) was measured by qRT-PCR using the $2^{-\Delta\Delta CT}$ method and endogenous *Gapdh* internal control. Figure is representative of one experiment.

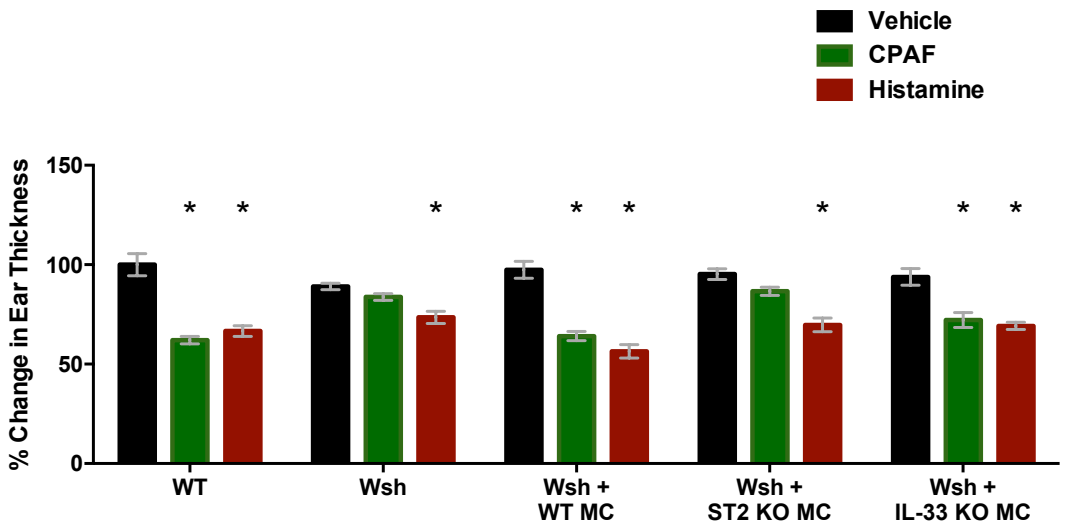


Figure 40. MC ST2, but not MC IL-33, is necessary for PAFR-mediated systemic immunosuppression.
WT, ST2 KO, or IL-33 KO MCs were transplanted into the dorsal skin of Wsh mice. Groups of 4-6 transplanted mice along with WT and Wsh were treated with vehicle, CPAF i.p. or histamine s.c. 5 days before sensitization to DNFB for CHS assay. CPAF does not confer immunosuppression in Wsh mice reconstituted with ST2 KO MCs. Error bars represent SEM. * Denotes statistically significant differences ($p<0.05$) in ear thickness measurements normalized to vehicle treated mice. Statistical significance determined using two-way ANOVA and the post-hoc Holm-Sidak method, with alpha=5%. Each row was analyzed individually, without assuming a consistent SD. Figure representative of one experiment.

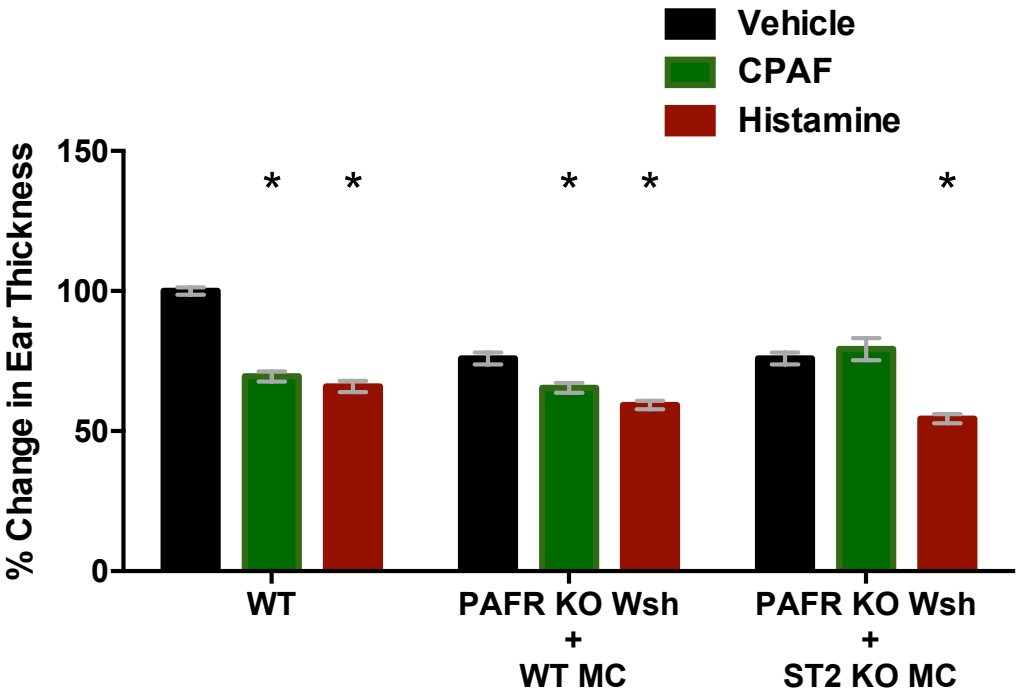
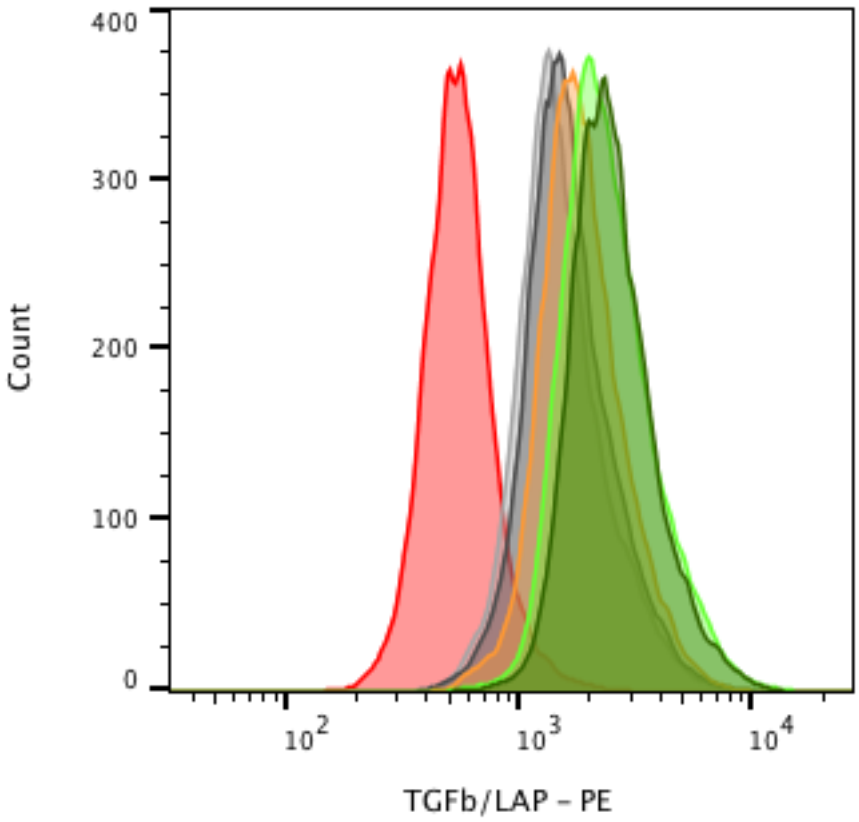


Figure 41. CPAF does not suppress CHS responses in PAFR KO Wsh mice reconstituted with ST2 KO MCs.
WT, or ST2 KO MCs were transplanted into the dorsal skin of PAFR KO Wsh mice. Groups of 6-12 transplanted mice along with WT were treated with vehicle, CPAF i.p. or histamine s.c. 5 days before sensitization to DNFB for CHS assay. CPAF does not confer immunosuppression in Wsh mice reconstituted with ST2 KO MCs. Error bars represent SEM. * Denotes statistically significant differences ($p<0.05$) in ear thickness measurements normalized to vehicle treated mice. Statistical significance determined using two-way ANOVA and the post-hoc Holm-Sidak method, with alpha=5%. Each row was analyzed individually, without assuming a consistent SD. Figure representative of one experiment.

As discussed previously, activation of MCs by IL-33 has been shown to mediate strong pro-inflammatory responses [162, 163]. This evidence along with previous CHS experiments, led to the hypothesis that MC ST2 activation might also initiate anti-inflammatory mechanisms, such as the induction of TGFβ. To test this hypothesis, MCs were incubated with IL-33 and flow cytometry analysis revealed that TGFβ expression is strongly upregulated by IL-33 as assessed by intracellular staining (Figure 42). Since, MC ST2 activation was found to induce anti-inflammatory mechanisms, exploring the mechanisms of how this pathway is involved in the suppressive mechanism of PAF proved to be important. Initial experiments found that ST2 is upregulated in MCs following PAFR activation (Figure 39B). These data helped to develop the hypothesis PAF and IL-33 may have some pharmacological interaction to induce the expression of anti-inflammatory mediators. Preliminary experiments show that PAF can potentiate IL-33 induced IL-10 expression, and synergize with IL-33 to induce COX-2 expression (Figure 43). However, PAF did not seem to have an effect on IL-33-induced IL-33 expression.



	SampleID	Mean : Comp-PE-A
	1000 ng/mL IL-33	2717
	100 ng/mL IL-33	2635
	10 ng/mL IL-33	2001
	1 ng/mL IL-33	1782
	0 ng/mL IL-33	1668
	Isotype	586

Figure 42. IL-33 upregulates TGFβ in MCs.
BMMCs were treated for 24hrs with increasing doses of IL-33. Cells were permeabilized with saponin and stained for TGFβ-bound LAP. Mean fluorescence are depicted in the table below. Figure is representative of three experiments

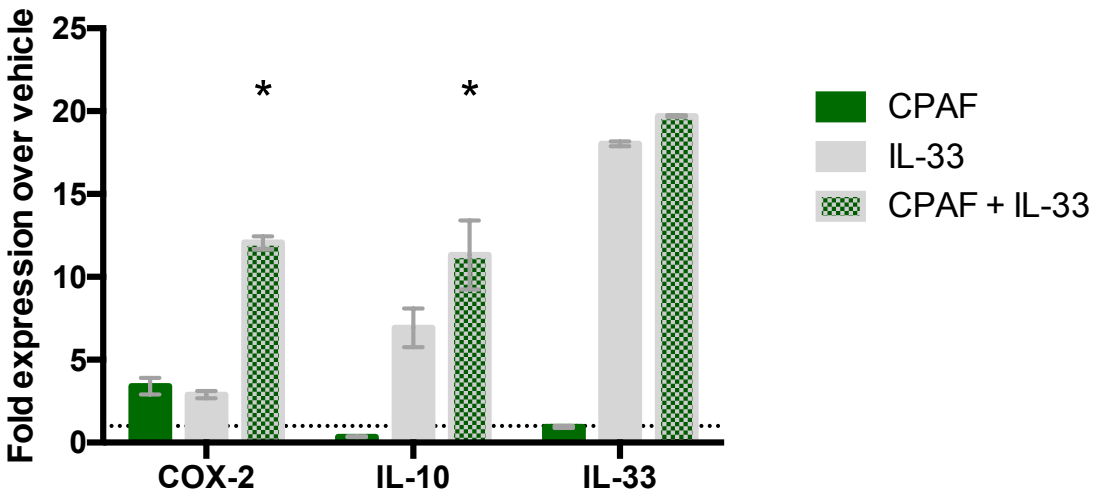


Figure 43. CPAF and IL-33 synergize to upregulate IL-10 and COX-2.
BMMCs were incubated with CPAF (100 nM) and IL-33 (10ng/mL) for 24hrs *(Ptgs2)* or 4hrs (*Il33* and *Il10*). Gene expression relative to vehicle (1-fold) was measured by qRT-PCR using the $2^{-\Delta\Delta CT}$ method and endogenous *Gapdh* internal control. Figure is representative of one experiment. Error bars represent SEM. * Denotes statistically significant differences ($p<0.05$) compared to CPAF and IL-33 treatment groups.

Chapter 4: Discussion and Future Directions

I. Chemotherapy induces systemic immunosuppression via the generation of PAFR agonists

The cytotoxic effects of chemotherapeutics pose a significant concern to patients. Despite narrow therapeutic concentrations, significant side effects pose a dosage limit to resistant tumors. Suppression of the immune system is a potential side effect of chemotherapy, which can promote opportunistic infections and diminish the effect of both host anti-tumor effects as well as exogenous immunotherapies [164-167]. While the mechanism of this suppression is controversial, some reports show that chemotherapy could induce a "rebound phase" via the activation of a chemotherapy induced "cytokine storm" [165, 168]. This might in part be the result of the bivalent pro-inflammatory and anti-inflammatory nature of PAFR signaling. I hypothesize that the effects of chemotherapy on the host immune system could be the result of PAFR agonists generated during chemotherapy that therefore mediate systemic immunosuppression. The approach to study this hypothesis is two fold: 1.) Test to see if chemotherapy treated melanoma generates PAFR agonists, and 2.) Test to see if chemotherapy induced PAFR agonists can promote tumor growth of a second tumor.

Using our specific calcium mobilization fluorometric assay involving PAFR-positive KBP cells, I found that chemotherapeutics, namely etoposide, cisplatin and melphalan, could generate PAFR agonists from mouse and human melanoma cells in vitro (Figures 7). These experiments revealed that incubating melanoma cells with chemotherapy generates the greatest concentration of PAFR agonists after two hours of incubation. Interestingly, this trend seemed to decrease after four hours of incubation,

possibly suggesting that the PAFR agonists being generated were being metabolized, or possibly that some of the ox-GPCs are inhibiting PAFR activation. In fact some of the ox-GPCs generated are partial agonists of the PAFR, which in high concentrations could decrease the response of full agonists.

Using a similar approach to measure PAFR agonists by calcium mobilization, PAFR-expressing melanoma cells (B16P) generated greater concentrations of PAFR agonists when treated with chemotherapeutics, dacarbazine, etoposide and cisplatin, as compared to PAFR-null melanoma (B16M) cells (Figure 8A). Additionally, pretreating melanoma cells with antioxidants NAC and vitamin C attenuated the generation of PAFR agonists by chemotherapy treatment (Figure 8B). In light of these data, this chemotherapy-induced generation of PAFR seems to be partially driven by both enzymatic, as well as, non-enzymatic PAF synthesis mechanisms. As antioxidants can attenuate the generation of these PAFR agonists (Figure 8B), this suggests that this process involves the oxidation of GPCs by ROS. Even with antioxidants, however, a significant amount of PAFR agonists were still detected, suggesting the presence of another mechanism that generates PAFR agonists. This could be the result of PAFR activation on melanoma cells (Figure 8A) or the activation of PLA_2/LPCAT via another mechanism following treatment with chemotherapeutics. More studies are necessary to describe this mechanism fully, but may involve the activation of apoptotic or necrotic pathways. Structural studies by Prof. Robert Murphy using mass spectrometry confirmed that chemotherapeutic agents generated both PAF as well as ox-GPC PAFR agonists (Figure 9). Still, the exact mechanism by which these PAFR agonists are generated requires further investigation.

Our group previously demonstrated that systemic PAFR activation by UVB or CPAF promotes tumor growth [25]. These observations led to the hypothesis that PAFR agonists generated as a result of chemotherapy could also promote tumor growth. The calcium mobilization functional assay, described above, demonstrated that lipid extracts from implanted tumors in mice treated with chemotherapy contained higher concentrations of PAFR agonists than those treated with vehicle (Figure 10). This generation of PAFR agonists in vivo was found to be blocked by feeding the mice an antioxidant diet consisting of vitamin C chow and NAC in water (Figure 10A). These data led to the hypothesis that chemotherapy induced generation of PAFR agonists in vivo could modulate the growth of a second tumor. To test this hypothesis, a dual tumor model was used, where one tumor was treated with intratumoral chemotherapy and the contralateral tumor was measured. Tumor measurements demonstrated that PAFR agonists generated by chemotherapy could promote growth of a contralateral tumor in WT tumor-burdened mice and not in PAFR-KO hosts (Figure 11 and 12). Feeding the mice an antioxidant diet, however, blocked the tumor growth promoting effect of chemotherapy (Figures 11C, 11D and 12B), suggesting that this promotion of tumor growth is in part due to the generation of ox-GPC PAFR agonists. Tumor measurements did not demonstrate significant tumor growth differences in tumors treated with intratumoral injections within a treatment group, yet some differences were observed between treatment groups. Namely, tumor growth differences in injected tumors between vehicle and chemotherapy-treated groups were found (Figure 13). In fact, decreased growth in contralateral (untreated) tumors that were measured was noted when comparing mice treated with chemotherapy versus vehicle (Figure 11B). This is likely the

result of chemotherapy acting appropriately both locally and systemically to attenuate tumor growth. The chemotherapeutics injected are likely not confined to the tumor volume, but rather diffusing systemically. Additionally, WT hosts promoted greater growth of a second tumor following intratumoral chemotherapy than PAFR KO hosts (Figure 11B), indicating that this increase in tumor growth is due to the effects of PAF.

Previous work in the field has demonstrated that the immunosuppressive effects of PAF are mediated by COX-2 derived prostaglandins and Tregs [24, 25, 36], and that the immunosuppressive effects of UVB are mediated by MCs [68, 73]. These lines of evidence led to the hypothesis that these chemotherapeutic agents may also be involved in mediating the suppressive effects of chemotherapy on the immune system. Using a single tumor model, COX-2 inhibitors blocked the increase in tumor growth driven by CPAF (Figures 14A and B). Notably, neither CPAF nor COX-2 inhibitors exerted any effects on tumor growth in PAFR KO mice (Figure 14C). Using a dual tumor model, the chemotherapy-induced promotion of tumor growth by melphalan and etoposide was also found to be blocked by COX-2 inhibitor SC-236 (Figure 15).

Interestingly, CPAF was found to increase intratumoral and draining LN Treg numbers in a process attenuated by COX-2 inhibitors (Figure 16ABC). These data led to the hypothesis that chemotherapy-induced promotion of tumor growth may be mediated by Tregs. To test this hypothesis a dual tumor model was used, where mice were treated with a Treg-depleting antibody cocktail prior to tumor implantation. Left tumors were treated with chemotherapy in these mice and the subsequent tumor growth measurements demonstrated that the tumor growth promoting effects of chemotherapy could be blocked by Treg depleting antibodies (Figure 16D). This evidence suggests that Tregs are

necessary to mediate chemotherapy-induced immunosuppression. Lastly, I sought to test the hypothesis that MCs are involved in the tumor growth-promoting effects of PAF. Using a single tumor model, MC-deficient Wsh mice were not found to be sensitive to the tumor growth promoting effects of CPAF (Figure 18), suggesting that MCs may be involved in PAFR-mediated increase in tumor growth.

The work presented here demonstrates that therapeutic doses of chemotherapeutic agents can induce the generation of PAFR agonists when treating cancer cells. In fact even humans, PAFR agonists were also found in perfusate samples from patients undergoing ILP melphalan chemotherapy (Figure 17). In collaboration with Prof. Robert Murphy, PAF and several PAFR agonistic ox-GPCs were found in the lipid extracts from these perfusates (Figure 17B). Chemotherapy appears to promote the oxidation of GPCs via the generation of ROS, and that these chemotherapy-induced PAF species promote growth of a second tumor by mediating systemic immunosuppression that is mediated by COX-2, Tregs, and MCs. These findings are consistent with recent published data that chemotherapy induces suppression of the immune system [164-167], and that PAF-induced systemic immunosuppression is mediated by prostaglandins and COX-2 [24, 25, 36].

As discussed previously, the immunosuppressive effects of chemotherapeutics is not a novel observation. In fact since its synthesis in 1953 by Bergel and Stock, melphalan, an alkalating analog, has displayed these characteristics during its use in treating leukemia and solid tumors [169]. Due to its structure as a phenylalanine-substituted mustard gas, melphalan is particularly effective against active tumors that use large amounts of phenylalanine such as melanoma. [169]. Originally, however, since

1988 melphalan has been used in autologous bone-marrow transplantation [169]. This use is largely due to well-described immunosuppression and bone-marrow ablation characteristics of high-dose melphalan, especially when administered intravenously [170]. The data in this manuscript support the hypothesis that chemotherapeutics such as etoposide, melphalan, dacarbazine, and cisplatin, induce systemic immunosuppression via the generation of PAFR-agonistic ox-GPCs in a process blocked by antioxidants, COX-2 inhibitors, and Treg-depleting antibodies (Figure 44). Still, more work is necessary to shed light on the mechanism of immune suppression by chemotherapy, where the data in this manuscript provides some light to the role of PAF in this mechanism. Part of this is the impetus for Part II, where the role of MCs in the mechanism of PAFR-mediated systemic immunosuppression is further investigated.

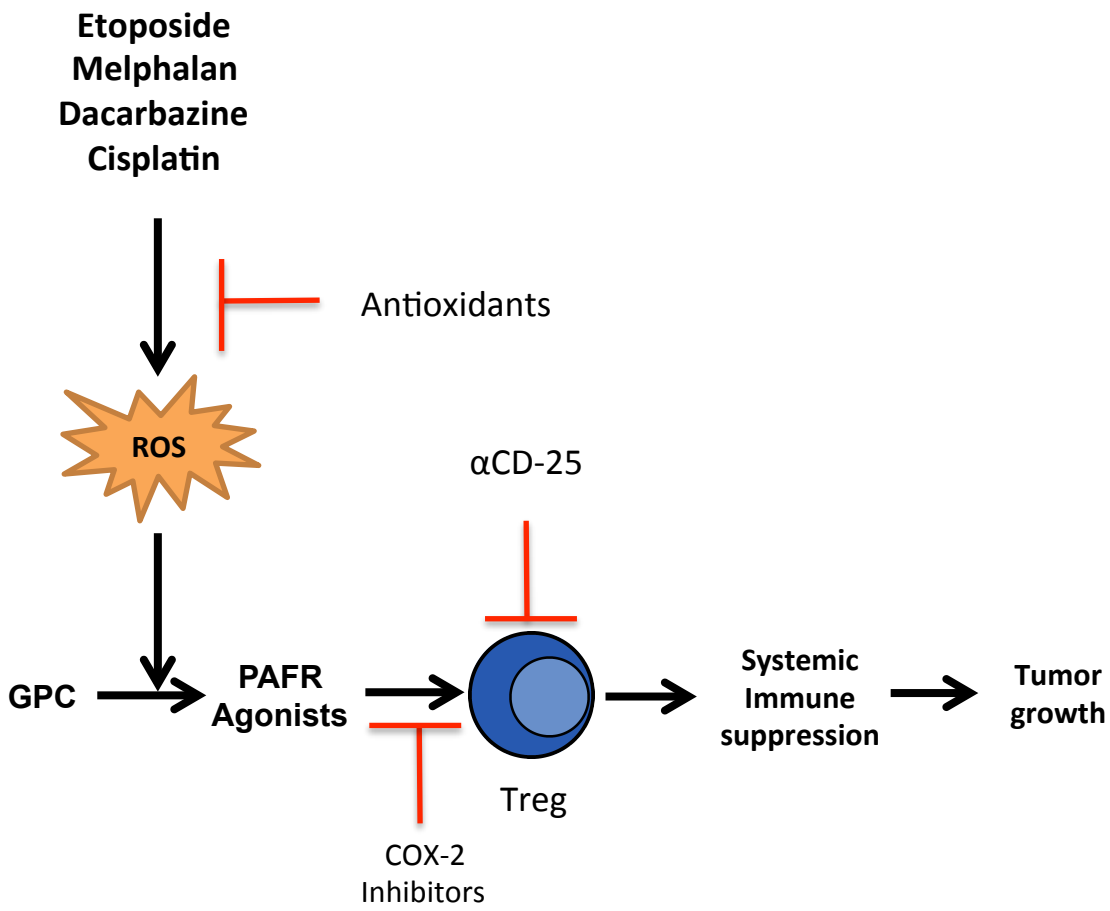


Figure 44. Current model for chemotherapy-induced PAFR-dependent increase in tumor growth.
Chemotherapy induces the generation of PAFR-agonistic ox-GPCs via the oxidation of GPCs by ROS. The generation of these PAFR agonists is blocked by antioxidants. Subsequent PAFR activation induces Treg-dependent increase in tumor growth via systemic immunosuppression. This increase in tumor growth can be blocked by COX-2 inhibitors and Treg-depleting antibodies.

II. Mast cell-derived histamine and prostaglandins mediate IL-33 receptor dependent PAF-induced immunosuppression

PAF and the activation of the 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 now chemotherapy [24, 25, 36, 140, 171]. Still, the mechanism of this suppression remains to be fully elucidated. While key players have been identified, namely COX-2, IL-10, Tregs and MCs among others to be uncovered, their interactions have not been thoroughly investigated. These findings shaped the hypothesis that dermal MCs may play a central role in initiating the suppressive effects of PAF on immune responses. To test this hypothesis, MC transplantation and CHS models were used to investigate the suppressive effects of PAFR stimulated dermal MCs on cell-mediated immunity. Initial experiments demonstrated that MCs derived from BM expressed a functional PAFR as measured by calcium mobilization (Figure 20). Additionally, reconstitution of dermal MCs in Wsh mice rescued the sensitivity to immunosuppression by UVB and CPAF absent in MC-deficient mice (Figure 21). These findings led to the hypothesis that the MC PAFR is necessary to mediate the immunosuppressive effects of PAF. To test this hypothesis, BMMCs from WT or PAFR KO mice were transplanted into Wsh mice. This experiment demonstrated that MC PAFR was necessary for CPAF to the attenuate ear swelling challenge response (Figure 22). Additionally, by reconstituting PAFR KO Wsh mice with WT MCs, MC PAFR activation was shown to be sufficient to suppress the CHS response (Figure 22). Notably, a similar number of MCs were found in reconstituted Wsh mice

compared to WT mice (Figure 23). Together, this evidence suggests that the MC PAFR is both necessary and sufficient to mediate PAF systemic immunosuppression.

It was previously shown that subcutaneous histamine injections could attenuate CHS responses in WT and MC-deficient mice [68]. In line with these observations, histamine was found to also suppress CHS responses in PAFR KO mice (Figure 24). Additionally, HDC KO mice appear to be sensitive to immunosuppression by histamine, but not CPAF (Figure 25). Together, these data suggest that histamine may promote systemic immunosuppression downstream of PAFR activation. MCs are one of the main contributors of histamine in inflammation. In fact, MCs are known to release large stores of histamine by rapid degranulation following FcεRI coupling, in a process involving intracellular calcium mobilization [172]. This thought process led to the hypothesis that MCs might release histamine following PAFR activation and that MC-derived histamine is an important mediator of PAF-induced immunosuppression. MC PAFR activation revealed upregulation of HDC mRNA and protein expression, and stimulation of histamine release (Figure 26). Surprisingly, transplantation of HDC KO MCs into Wsh mice still rescued the immunosuppressive effects of CPAF, but not if HDC KO MCs were transplanted into Wsh mice lacking PAFR (Figure 27). This evidence suggests that MC histamine release plays a role in PAFR-mediated systemic immunosuppression, but that there are other compensatory pathways due to the expression of PAFR on other cells (e.g. basophils, keratinocytes, fibroblasts, endothelial cells). Still, it seems that MC PAFR is necessary, but perhaps activation of other PAFR expressing cells contribute to reaching the threshold necessary to suppress immune responses. For example, it has been

previously shown that PAFR activation on keratinocytes upregulates COX-2 expression [130] and treatment with CPAF upregulates IL-33 expression in fibroblasts [161].

As previously mentioned, COX-2 has been shown to be involved in mediating the immunosuppressive effects of UVB and PAF. Furthermore, PAFR activation has been shown to upregulate COX-2 [130]. In line with this evidence, PAFR activation also upregulates COX-2 in MCs (Figure 29) and MC COX-2 expression appears to be necessary for PAFR-mediated systemic immunosuppression as found by CHS (Figure 30). Additionally, COX-2 inhibitors were shown to block CPAF and histamine immunosuppression in WT mice (Figure 28), suggesting that COX-2 is involved downstream of PAF and histamine receptor activation. One of the proposed mechanisms for the involvement of COX-2 in this pathway is the interaction of prostaglandins and the regulation of cell chemotaxis. In particular, PGE_2 has been shown to mediate immunosuppressive responses by promoting chemotaxis of anti-inflammatory cells, while attenuating the attraction of pro-inflammatory cells [158]. One such example of this is the ability of PGE_2 to induce CXCL12 expression in endothelial cells [73]. Additionally, the Ullrich laboratory has shown that MC PAFR activation upregulates CXCR4 expression in MCs and promotes a necessary chemotaxis to draining LNs to mediate immunosuppression [73]. These findings led to the hypothesis that MC CXCR4 might be under the control of prostaglandins released by PAFR activation. To test this hypothesis, Wsh mice transplanted dorsally with CD45.1 WT BMMCs were treated with CPAF or vehicle, and SC-236 or vehicle. Histological (Figure 33) and flow cytometry (Figure 34) analysis of inguinal LNs in these mice revealed that CPAF induced the migration of MCs to LNs in a process blocked by COX-2 inhibitors. Furthermore, COX-

2 inhibitors were found to block the upregulation of CXCR4 by PAFR activation in MCs (Figure 35B), and PGE_2 receptor, EP4, activation was found to independently upregulate MC surface CXCR4 expression (Figure 35C). Lastly, it remains to be fully elucidated how the CXCL12 chemokine gradient is established to facilitate PAF-induced MC migration to draining LNs. In light of evidence that MC mediators can travel down lymphatic vessels to affect the LN microenvironment [81], PGE_2 can upregulate CXCL12 LNs [73], and that histamine can promote prostaglandin release [159, 160], led to the hypothesis that histamine released following MC PAFR activation could subsequently promote a CXCL12 chemokine gradient by upregulating COX-2 in draining LNs. Preliminary data suggested that subcutaneous histamine injection can increase COX-2 levels in inguinal LNs (Figure 36). These data support the hypothesis that histamine released following MC PAFR activation could promote COX-2 expression to provide the source of prostaglandins necessary to mount a CXCL12 chemokine gradient. Together this evidence suggests that the role of prostaglandins, and possibly both MC-derived prostaglandins and histamine, are important to mediate PAF-induced systemic immunosuppression and it may do so in part by regulating MC CXCR4 and LN COX-2 expression.

MC CXCR4 has been shown to be important for UVB-induced systemic immunosuppression, because MC migration to LNs via this chemokine pathway has been shown to be necessary [73, 74]. MC migration to LNs is not a new finding as MCs have been shown to migrate to LNs after various inflammatory stimuli [72]. While the role of MCs in the LN is a current topic of further investigation, it seems that MCs can be a pivotal player in directly influencing lymphocyte activation. Once in the LN, MCs could

release prostaglandins, histamine, and TGFβ, amongst other mediators, to influence lymphocyte activation [173], or recruit other immunosuppressive cell types [91, 93, 96, 174]. While CPAF and histamine-induced systemic immunosuppression could be blocked by TGFβ inhibitors (Figure 31), MC PAFR activation failed to induce TGFβ release (Figure 32). Still, basal TGFβ or histamine release could become relevant once MCs enter draining LNs. The immunosuppressive effect of MCs in the local microenvironment has been observed before [88]. However, MCs can upregulate TGFβ expression in response to IL-33, which seems to be involved in PAFR-mediated systemic immunosuppression.

The alarmin IL-33 has been shown to be released as a result of cell damage and to initiate immune responses. Recently, it was shown to be upregulated in skin irradiated with UVB and upregulated in dermal fibroblast after CPAF treatment [161]. These data led to the hypothesis that IL-33 may be important for PAFR-mediated systemic immunosuppression. Initial experiments demonstrated that mice lacking the IL-33 receptor (ST2), were sensitive to systemic immunosuppression by UVB and histamine, but had a decreased response to CPAF (Figure 37). Further experiments showed that compared to WT mice, ST2 KO mice had a decreased, but not significant, response to CPAF (Figure 38), and that WT BMMCs express ST2 that can be upregulated by treatment with CPAF and PGE_2 (Figure 39). Next, MC ST2, but not MC IL-33 was found to be necessary to mediate PAF-induced immunosuppression (Figure 40 and 41). Moreover, BMMCs treated with IL-33 were found to upregulate TGFβ expression (Figure 42), and IL-33 appears to synergize with CPAF to upregulate IL-10 and COX-2 expression (Figure 43). This evidence suggests that ST2 might be important in the

promotion of immunosuppression by CPAF, but that UVB activates other pathways that do not involve IL-33 signaling. For example, cis-UCA could be mediating the immunosuppressive effects of UVB in a process that does not require IL-33 signaling. In the context of PAF, however, IL-33 might be mediating systemic immunosuppression through the upregulation of IL-10, TGFβ, and COX-2, which have been implicated in UV-induced systemic immunosuppression. Still, the exact source of IL-33 relevant to this mechanism is a topic of current investigation. In line with previous observations, however, it appears that ST2 KO mice are still sensitive to immunosuppression by histamine, suggesting that histamine might mediate suppression of the immune system downstream of ST2 and PAFR.

These results suggest that COX-2, IL-33, and histamine mediate the immunosuppressive effects of PAF (Figure 45). 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. Of particular interest, would be to identify the site of action of histamine, PGE_2, and IL-33. The current evidence in this work gives rise to two hypotheses for how MCs may be mediating immune suppression: 1.) Either by direct interactions with lymphocytes in draining LNs following CXCR4-dependent MC migration to LNs, or 2.) 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 has been shown to be necessary to mediate systemic immunosuppression, and that this migration is mediated via CXCR4 chemotaxis following MC PAFR activation [73, 74, 127]. It has also been shown that MC-derived

particles can travel via lymphatic vessels to signal to distant LNs [81]. This could be important because histamine and prostaglandins released following MC PAFR activation could travel to draining LNs to influence T cell activity. Additionally, one of the main APCs in the skin, dendritic cell migration has been shown to be repressed by PGD_2 but promoted by PGE_2 [154, 155, 157, 175-178]. The migration of DCs could potentially play a role, because the downregulation of DC activity or migration in the skin could also account for decreased CHS responses by virtue of decreasing presentation of antigen during sensitization. Thus, the possibility exists that these two mechanisms contribute to the immune suppressive response of PAF, MC migration-dependent and –independent mechanisms.

Additionally, the kinetics of MC histamine and PGE_2 release may be relevant to study further. MC PAFR activation seems to induce early histamine release within an hour and PGE_2 release within 8 hours of activation, which may be relevant in the skin or in LNs provided that these mediators can flow to draining LNs. Conversely, mRNA and protein expression of HDC and COX-2 are upregulated after 12-24 hours. The expression timeline of this increase in protein expression more closely correlates with the timeline of MC migration to LNs following MC PAFR activation. These observations suggest that MC histamine and prostaglandin release could become relevant in the LN following MC migration. Thus, histamine and PGE_2 seem to have both early and late effects. Furthermore, it remains to be further elucidated where in the mechanism IL-33 is relevant.

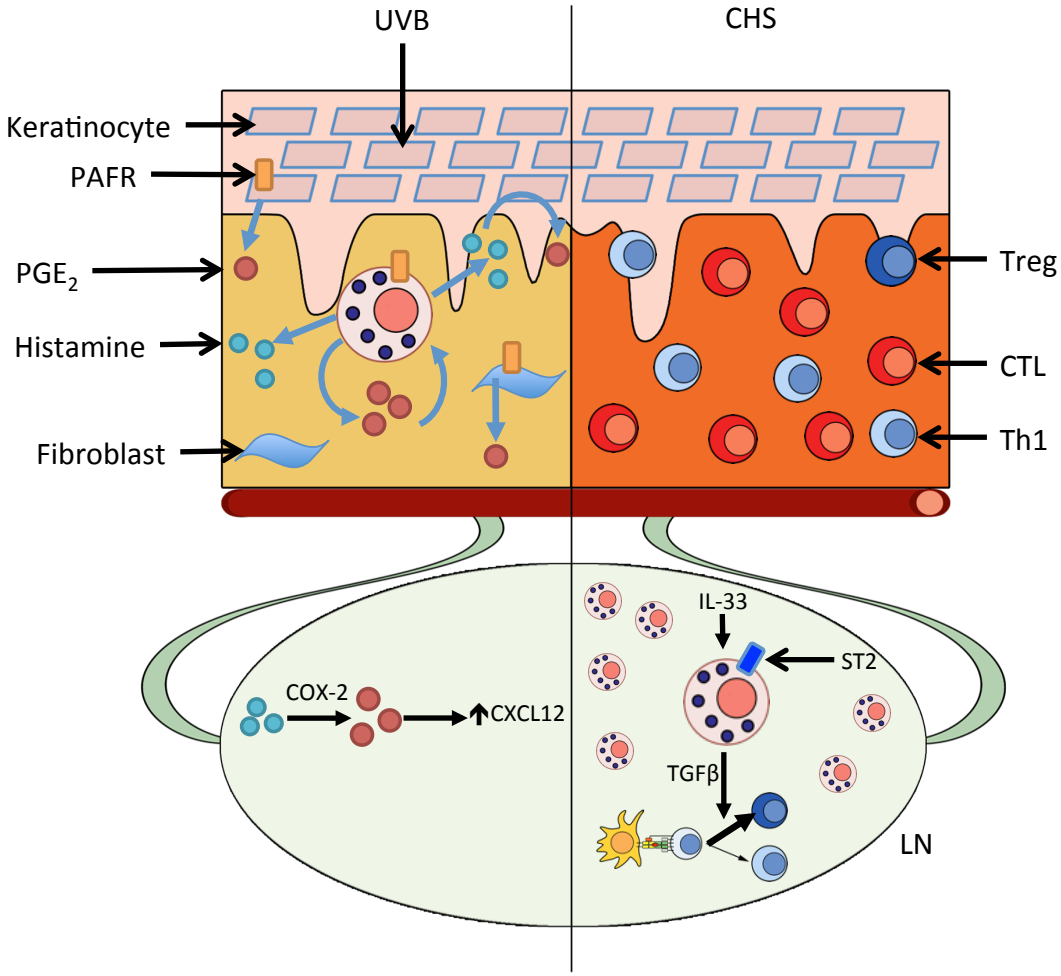


Figure 45. Current model of the role of MCs in PAFR-mediated systemic immunosuppression.
MC PAFR activation stimulates PGE_2 and histamine release that reactivate MCs via possible paracrine mechanisms to upregulate MC CXCR4. Additionally, histamine and PGE_2 can flow to draining lymph nodes to upregulate lymph node CXCL12 expression to provide the chemokine gradient for CXCR4-mediated MC migration to draining lymph nodes over the course of 24 hours. In the LNs, MCs induce Treg-mediated systemic immunosuppression, possibly by promoting Treg differentiation via the release of TGFβ subsequent to IL-33 stimulation. This immunosuppression is mediated by Tregs, partly by the decrease in Th1 populations, and resulting decreased CHS reactions.

III. Future Directions

To further dissect the role of MC migration in the suppressive effects of PAF on the immune system, I plan to investigate the role of MC COX-2 and histamine in PAFR-induced MC migration. Preliminary studies have demonstrated that COX-2 inhibitors can block the migration of MCs to draining LNs. Suggesting the importance of COX-2 derived prostaglandins in this mechanism. I also have some data suggesting that histamine and PGE_2 can have autocrine effects in MCs, suggesting that that MC COX-2 and histamine may be important for PAF-induced migration of MCs to draining LNs. To study this, I plan on studying underlying autocrine effects of PGE_2 and histamine in vitro, as well as the ability of COX-2 and HDC KO MC to migrate to draining LNs following PAFR activation. Additionally, I plan to investigate the role of prostaglandins released after MC PAFR activation on DC migration and subsequent changes in T cell activation. Lastly, moving forward with the hypothesis that MCs migrate to draining LNs, I want to test the role of MC PAFR activation on T cell differentiation. Previously, unstimulated MCs have been shown to promote Treg differentiation when cultured in vitro [179]. Still, the effects of histamine on Tregs is controversial, where some evidence suggests that histamine represses [92, 180] and promotes [145, 181] Treg function. Thus, I want to test the hypothesis that PAFR-activated MCs can promote the differentiation of Tregs in LNs by co-culture.

The mechanistic role of MCs in the immunosuppression of chemotherapy is not well elucidated. While dermal MCs could be conceivably activated by PAF-species made by chemotherapy, which MCs and how the MCs are activated in the context of tumor immunity are still a topic of investigation. Aside from the role of MCs, it remains to be

elucidated whether the suppression of the immune system by PAF is antigen specific. To investigate this further, I propose to make use of OVA-expressing melanoma cells and OT-I mice, whose majority of the T cell receptor repertoire is specific for the recognition of the OVA antigen. For these experiments, antitumor immunity can be modeled by transplanting OT-I T cells into mice burdened with OVA-expressing tumors. The tumor modulating effects of chemo and CPAF can then be studied in the context of antitumor immunity. Using this tumor model, the immunosuppressive effects of PAF on the adaptive immune response, versus non-specific anti-tumor immunity (such as NK cell and macrophages) can be investigated.

While pharmacokinetics and pharmacodynamics of melphalan treatment in humans have been carried out [169, 170], further studies are needed to uncover where PAF may be relevant in the immunosuppressive effects of chemotherapy, particularly etoposide and melphalan. For instance, it remains to be elucidated whether the PAFR agonists generated by chemotherapy are only formed by intratumoral chemotherapy or if systemic chemotherapy will also produce similar results. Perhaps, the generation and action of these PAFR agonists is confined to the tumor microenvironment. Moreover, it remains to be known if COX-2 inhibitors may be effective at reducing immunosuppressive effects of chemotherapy in humans. Further clinical studies aimed at investigating the efficacy of chemotherapy in patients taking COX-2 inhibitors would be necessary.

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Curriculum Vitae

Jesus A Ocana

Education

Kent State University
Chemistry, BS (Honors)
Biology, Minor
2006-2010

Indiana University
PhD in Pharmacology, July 2016
MD (In Progress)
Expected Graduation Date 2018

Personal Statement
From an early stage in my career development, I have held science with high regard and great curiosity. Now on my third year of graduate work, I have successfully contributed to several manuscripts, and am co-first author on a manuscript recently accepted in Cancer Research. I look forward to the sense of fulfillment with the publication of my dissertation research and completion of my MD and PhD degrees. In the years to come, I plan to manage a well-funded laboratory in the field of immunopharmacology, while still maintaining part-time clinical duties. I strive to contribute to the scientific community in my efforts as a successful physician scientist.

Extracurricular Activities
Pharm & Tox- Graduate Student President
Rock for Riley – Former President of Philanthropy
Competency IX - Philanthropic Medical School Band
Combined Degree Student Council – Former Co-Chair
Operation Leftover- Volunteer to cater to the homeless of Indianapolis

Publications
Bunge SD, **Ocana JA**, Cleland TL, Steele JL. Synthetic, structural, and theoretical investigation of guanidinate complexes containing planar Cu_6 cores. ***Inorg Chem.*** 2009 Jun 1;48(11):4619-21. PMID: 19388654.

Ocana JA, Bunge SD. Investigation of Guanadinate Group 11 Complexes and their stabilization with Silyl and Alkyl Amines. Senior Honors Thesis, Kent State University, 2010

Sahu RP, Turner MJ, DaSilva SC, Rashid BM, **Ocana JA**, Perkins SM, Konger RL, Touloukian CE, Kaplan MH, Travers JB. The environmental stressor ultraviolet B radiation inhibits murine antitumor immunity through its ability to generate platelet-activating factor agonists. ***Carcinogenesis.*** 2012 Jul;33(7):1360-7. PMID: PMC3405652.

Sahu RP, Petrache I, Van Demark MJ, Rashid BM, **Ocana JA**, Tang Y, Yi Q, Turner MJ, Konger RL, Travers JB. Cigarette Smoke Exposure Inhibits Contact Hypersensitivity via the Generation of Platelet-Activating Factor Agonists. ***J Immunol.*** 2013 Mar 1;190(5):2447-54. PMID: PMC3577966.

Adas SK, **Ocana JA**, Bunge SD. Synthesis and Structural Characterization of a Series of Group 11 2,2-Dialkyl-1,3-dicyclohexylguanidinate Complexes. ***Aust J Chem.*** 2014 Jun: 67:1021–1029.

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Rezania S, Sahu RP, **Ocana JA,** DaSilva SC, Bradish JR, Warren SJ, Rashid B, Travers JB, and Konger RL. Topical application of a platelet activating factor receptor agonist suppresses phorbol ester-induced acute and chronic inflammation and has cancer chemopreventive activity in mouse skin. ***PLOS ONE.*** 2014 Nov: 9 (11): e111608. PMID: 4222871.

Sehra S, Serezani AP, **Ocaña JA**, Travers JB, Kaplan MH. Mast Cells Regulate Epidermal Barrier Function and the Development of Allergic Skin Inflammation. ***Journal of Investigative Dermatology.*** 2016 Mar. PMID: 27021404.

Presentations

Ocana, J. A., Bertke, J., Cleland, T. and Bunge, S. D., "**Synthesis and Characterization of a Novel Series of Hetero-Ligated Lanthanide Compounds for the Ring Opening Polymerization of ε-Caprolactone.**" Poster: Ohio Science and Engineering Alliance Symposium at Wright State University, American Chemical Society Regional Meeting at Columbus, OH. Talk: Research Night at Kent State University.

Ocana, J. A., Steele, J., Cleland, T. and Bunge, S. D., "**A Synthetic and Theoretical Investigation of Cyclic Multinuclear Group 11 Guanadinate Complexes**" Poster: Ohio Inorganic Weekend at University of Toledo (November 2008). Talk: Ohio Science and Engineering Alliance Symposium at Ohio State University.

Ocana, J.A., Sahu, R.P., Harrison, K.A., Touloukian, C.E., Sun, L., Murphy, R.C., Konger, R.L., and Travers, J.B., "**The dark side of chemotherapy: Evidence that chemotherapeutic agents generate Platelet-activating Factor agonists that subvert tumor immunity."** Poster**:** Indiana University Simon Cancer Center: Cancer Research Day 2012. Poster: National MD/PhD Student Conference, Keystone, Co.

Ocana, J.A., Yao, Y., Sahu, R.P., Konger, R.L., and Travers, J.B., **"The role of mast cells in Platelet Activating Factor-mediated systemic immunosuppression."** Oral & Poster: Autumn Immunology Conference, Chicago, 2013. Poster: Bioactive Lipids in Cancer, Inflammation and Related Diseases, Puerto Rico, 2013.

Ocana, J.A., Yao, Y., Sahu, R.P., Konger, R.L., and Travers, J.B., **"Platelet Activating Factor-mediated systemic immunosuppression requires IL-33 and various mast cell mediators."** Oral Plenary & Poster: Society of Investigative Dermatology, Albuquerque, 2014.
Ocana JA, Yi Q, Yao Y, Bryce P, Kaplan M and Travers JB. **"Mast cell-derived histamine is necessary for Platelet Activating Factor mediated systemic immunosuppression."** Oral & Poster: American Association of Immunologists Annual Meeting, New Orleans, 2015.

Honors Awards and Fellowships

Access Scholarship, Kent State University, 2006-2010
Oscar Ritchie Memorial Scholarship, Kent State University, 2006-2010
Honors College Scholarship, Kent State University, 2006-2010
Choose Ohio First Scholar, Kent State University, 2008-2010
Golden Key Honors Society, Kent State University, 2008-2010
Student Poster Presentation and Contest: Award of Excellence, Kent State University, 2009
Undergraduate Student Award in Analytical Chemistry, Kent State University, 2009
Undergraduate Student Award in Analytical Chemistry, American Chemical Society, 2009
Senior Honors Thesis Fellowship, Kent State University, 2009-2010
Lubrizol Chemistry Scholarship, Kent State University, 2010
Summa Cum Laude, Kent State University, 2010
IU Simon Cancer Center Poster Presentation: Honorable Mention, IUSM, 2013
Merilyn Hester scholarship recipient, IUSM, 2013
Cancer Biology Training Program Fellowship, IUSM, 2013
John Wallace Minority Scholarship, Autumn Immunology Conference Council, 2013
Annual MD/PhD National Student Conference Diversity Travel Award, Keystone, CO, 2013
Ruth L. Kirschstein F30 National Research Service Award, Submitted, 2013
IUSM Travel Grant, IUSM, 2014
Immunology and Infectious Disease Training Program T32-Trainee Award, IUSM, 2014
Ruth L. Kirschstein F31 National Research Service Award, Submitted, 2014
IUSM Travel Grant, IUSM, 2015
R. R. Paradise Travel Award, Department of Pharmacology, IUSM, 2015
IMMUNOLOGY 2015 Trainee Abstract Travel Award, 2015
FASEB MARC Program Poster/Oral Presentation Travel Award, 2015
President's Diversity Dissertation Fellowship, Indiana University, Indianapolis, 2015
K. K. Chen Fellowship, Department of Pharmacology, IUSM 2015