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Topical photodynamic therapy induces systemic immunosuppression via generation of platelet-activating factor receptor ligands

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Photodynamic therapy (PDT) is an FDA-approved procedure used for the treatment of pre-cancerous actinic keratosis as well as superficial skin cancers (Morton et al, 2008). The treatment is based on the topical application of a photosensitizing agent or its metabolic precursor (e.g. 5-aminolevulinic acid; 5-ALA) that has preferential uptake by proliferative/ metabolically active cells (e.g. malignant cells), followed by exposure of the treated skin to a light source of a specific wavelength. This exposure promotes the photosensitizing agent to generate singlet oxygen and then other reactive oxygen species, leading to oxidative stress and cell death (Dougherty et al, 1998). Several studies have shown that PDT can cause immunosuppression in both humans and mice, but the mechanisms underlying these effects are not totally clear (Matthews and Damian, 2010, Mroz and Hamblin, 2011).

Platelet-activating factor (1-alkyl-2-acetyl glycerophosphocholine; PAF) is a lipid-derived mediator with diverse functions. Glycerophosphocholines (GPCs) from cell membranes can undergo oxidation resulting in the formation of oxidized GPCs (Ox-GPCs) which can act as potent agonists for the PAF receptor (PAF-R) (Konger et al, 2008). Numerous

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environmental pro-oxidative stressors from cigarette smoke to ultraviolet B radiation (UVB) can induce systemic immunosuppression via generation of Ox-GPC PAF-R ligands (Sahu et al, 2013, Walterscheid et al, 2002, Wolf et al, 2006, Yao et al, 2009, Zhang et al, 2008). Of interest, UVB-generated PAF-R ligands also augment experimental melanoma tumor growth by suppressing anti-tumor immunity (Sahu et al, 2012). Apoptotic cells also express PAF-R ligands and were shown to promote the growth of a sub-tumorigenic inoculum of melanoma cells (Bachi et al, 2012). The present study was designed to test the hypothesis that PAF-R activation mediates PDT-induced systemic immunosuppression.

First, to evaluate whether PDT produces PAF-R ligands, we simulated PDT in the human keratinocyte-derived cell line HaCaT by incubating the cells with 5-ALA (1 mM, 4 h) and exposed them to a blue light (415 nm, 10–20 J/cm^2) source. As multiple glycerophosphocholine species can act as PAF-R agonists, we quantified **total** PAF-R biochemical activity using PAF-R-expressing KBP cells that produce IL-8 when the receptor is activated (Pei et al, 1998). KBP and PAF-R-negative KBM cells were exposed for 6 h to lipid extracts from HaCaT cells treated with 5-ALA, and exposed to blue light (PDT). The IL-8 production was expressed as the % of normalized lipid extract IL-8 response versus that induced by 100 nM of the metabolically stable PAF-R agonist 1-hexadecyl-2-*N*-methylcarbamoyl glycerophosphocholine (CPAF; see supplementary Fig S1 for example of CPAF dose-response curve). As shown in Fig. 1A, 5-ALA plus blue light generated significant levels of PAF-R ligands, with no perceptible effect of 5-ALA or light treatment alone. The levels of PAF-R ligands remained elevated for at least 1h post PDT (Fig. 1B). Moreover, lipid extracts from PDT-treated HaCaT cells also induced intracellular calcium mobilization responses in KBP cells loaded with the calcium-sensitive dye Fura-2 AM, whereas lipid extracts from sham-treated HaCaT cells resulted in a negligible response (see Supplemental Fig. S2). However, lipid extracts from PDT-treated HaCaT cells did not induce IL-8 production (*data not shown*) nor intracellular calcium mobilization responses in PAF-R-negative KBM cells (Fig. S2). To structurally define the PAF-R ligands generated by PDT in HaCaT cells, we used mass spectrometry with deuterium-labelled internal standards as per our previously published methodology (Yao et al, 2012). As shown in Fig. 1C, we noted approximately three-fold increased levels of *sn-1* C-16 and C-18 PAF species in PDT-treated cells. However, unlike other classic pro-oxidative stressors such as UVB, PDT did not identify increased levels of Ox-GPCs (see Supplemental Methods for all GPC species monitored). These findings suggest that PDT-generated PAF-R ligands are enzymatically produced, not via ROS-mediated non-enzymatic processes. Consistent with this result, pretreatment of HaCaT cells with antioxidants vitamin C or N-acetylcysteine at doses that attenuate UVB-generated PAF-R agonists (Yao et al., 2012) did not block PDT-generated PAF-R agonistic activity (see Supplemental Fig. S3).

Studies have shown that topical PDT induces systemic immunosuppression (Hayami et al, 2007). Since PDT induces production of PAF *in vitro*, we assessed whether PDT-induced systemic immunosuppression is via PAF-R engagement. To this purpose we used a well-established model of contact hypersensitivity (CHS) to the chemical dinitrofluorobenzene (DNFB) using WT and *Ptafr*−/− mice in studies approved by our institution's animal review committee (see Zhang et al., 2008 for methods). PDT was performed by adding 5-

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ALA (20 mg/mouse) to the shaved lower back of the mice. After 4 h (in the dark), the mice were anesthetized and part of the shaved area on the lower back exposed to a blue light (20 J/cm^2). Five days after PDT, the mice were sensitized with DNFB topically applied to the shaved non-PDT-treated upper back (to test for systemic immunosuppression) and challenged 9 days later with DNFB applied to the ears. The intensity of the immune response to DNFB was measured by change in the ear thickness prior and 24 h after challenge. As positive controls for immunosuppression, one group was injected with CPAF (250 ng/mouse, i.p.) and other injected with histamine (250 μg/mouse, s.c.) five days prior to sensitization with DNFB. As shown in Fig. 2, PDT significantly inhibited CHS reactions in WT, but not in PAF-R-deficient mice. Injection of CPAF had the same effect as PDT, inducing immunosuppression only in WT mice. Similar to what we observed *in vitro*, 5-ALA only and light only were not able to inhibit CHS reactions in WT mice as observed for PDT (5-ALA + light) (*data not shown*).

Together, these results show that PDT induces the local generation of PAF which leads to systemic immunosuppression. The mechanisms involved are yet to be described, but studies suggest the involvement of cyclooxygenase-2 (COX-2)/PGE_2, mast cells, regulatory T cells and IL-10 in PAF-R-mediated systemic immunosuppression (Sahu et al., 2012, Sahu et al., 2013, Walterscheid et al., 2002, Wolf et al., 2006, Zhang et al., 2008). The finding that PDT induces systemic immunosuppression via PAF-R signaling could provide the impetus for testing the ability of inhibitors of this pathway (e.g., COX-2 inhibitors) to improve the effectiveness or limit the side effects of this therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

PDT	photodynamic therapy
5-ALA	5-aminolevulinic acid
CHS	contact hypersensitivity
WT	wild type
PAF-R KO	PAF-R-deficient mice
KBP	PAF-R-expressing human epithelial KB cell line
KBM	PAF-R-non-expressing human epithelial KB cell line
COX-2	cyclooxygenase type 2

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PGE_2	prostaglandin E_2
UVB	ultraviolet B
IL-8	interleukin-8
IL-10	interleukin-10
CPAF	1-hexadecyl-2-*N*-methylcarbamoyl glycerophosphocholine
DNFB	dinitrofluorobenzene
NAC	N-acetylcysteine
PAF	platelet-activating factor
GPC	glycerophosphocholine
Ox-GPC	oxidized GPC
PAF-R	PAF receptor
i.p	intraperitoneal injection
s.c	subcutaneous injection

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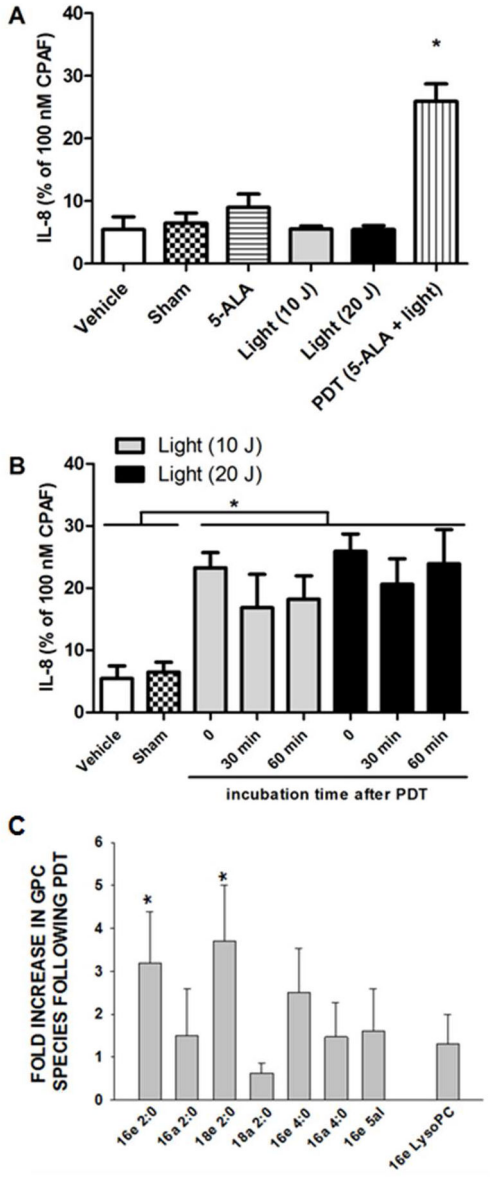

Figure 1. PDT induces PAF-R ligand formation in human HaCaT keratinocytes

(a) HaCaT cells were incubated with *5-ALA* followed by exposure to a blue LED *light* source alone (10 or 20 J/cm^2) or both 20J/cm^2 + 5-ALA (*PDT*). Controls consisted of HaCaT cells exposed to 5-ALA alone; to blue light alone; or to the lipid extract vehicle (ethanol). Lipid extracts were obtained immediately following treatment and normalized to cell number (2.5×10^6 cells), then added to KBP cells. After 6 h, IL-8 was quantified as a measure of PAF-R agonistic activity. One group of KBP cells was treated with 100 nM CPAF as a positive control and the other group with 0.5% ethanol vehicle. **(b)** For the time course analysis of PDT-generated PAF-R ligand formation, after PDT (10 or 20 J/cm^2), cells were incubated for 0, 30 or 60 min at 37°C and lipid extracts obtained and IL-8 levels compared to sham treated cells for 60 min. Results in (a) and (b) are expressed as the percentage of IL-8 relative to amounts induced by CPAF. In **(c)**, lipid extracts from Sham-

versus PDT-treated HaCaT at time 0 were analyzed by liquid chromatography-mass spectrometry and expressed as fold-increase relative to sham. The data are mean ± SE from at least 3 independent experiments. * denotes statistically significant ($p < 0.05$) changes from vehicle or sham.

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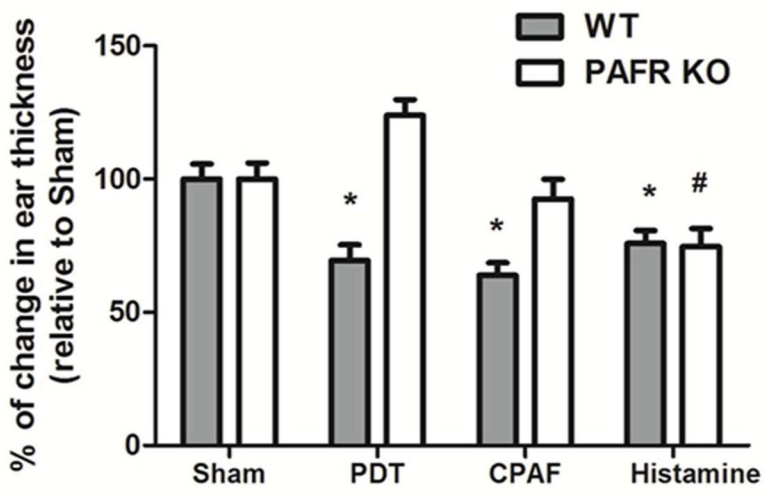

Figure 2. PDT inhibits CHS to DNFB in a PAF-R-dependent manner

For PDT treatment, groups of five to eight WT and PAF-R KO (*Ptafr*−/−) mice were treated topically with 5-ALA in the shaved lower back. After 4 h, the shaved lower back area was exposed to blue light (20 J/cm^2). Other groups of mice were injected with CPAF (250 ng/mouse, i.p.) or histamine (250 μg/mouse, s.c.). Five days after treatments, shaved upper back of all mice was painted with DNFB. After 9 days, the ear thickness was measured, one ear treated with DNFB the other with vehicle, and measured again after 24 h. Results are mean ± SE percentage of change in the ear thickness relative to sham group from three separate experiments using a minimum of 5 mice per experimental group. * and $^{\#}$ denotes statistically significant ($p < 0.05$) changes from sham (* for WT and $^{\#}$ for PAF-R KO).