The environmental stressor ultraviolet B radiation inhibits murine antitumor immunity through its ability to generate platelet-activating factor agonists

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Ubiquitous pro-oxidative stressor ultraviolet B radiation (UVB) to human or mouse skin generates platelet-activating factor (PAF) and novel oxidatively modified glycerophosphocholines (Ox-GPCs) with PAF-receptor (PAF-R) agonistic activity. These lipids mediate systemic immunosuppression in a process involving IL-10. The current studies sought to determine the functional significance of UVB-mediated systemic immunosuppression in an established model of murine melanoma. We show that UVB irradiation augments B16F10 tumor growth and is dependent on host, but not melanoma cell; PAF-R-expression as UVB or the PAF-R agonist, carbamoyl PAF (CPAF), both promote B16F10 tumor growth in wild-type (WT) mice, independent of whether B16F10 cells express PAF-Rs, but do not augment tumor growth in Pafcr−/− mice. UVB-mediated augmentation of experimental murine tumor growth was inhibited with antioxidants, demonstrating the importance of Ox-GPC PAF-R agonists produced non-enzymatically. Host immune cells are required as CPAF-induced augmentation of tumor growth which is not seen in immunodeficient NOD SCID mice. Finally, depleting antibodies against IL-10 in WT mice or depletion of CD25-positive cells in FoxP3−/− mice block UVB and/or CPAF-induced tumor growth supporting a requirement for IL-10 and Tregs in this process. These findings indicate that UVB-generated Ox-GPCs with PAF-R agonistic activity enhance experimental murine melanoma tumor growth through targeting host immune cells, most notably Tregs, to mediate systemic immunosuppression.

Introduction

Malignant melanoma is the seventh most common type of cancer (1,2). The incidence of melanoma is increasing more than any other malignancy in the USA (1,2). The American Cancer Society estimates over 68,000 new patients diagnosed with melanoma and 8700 died from malignant melanoma in 2010 (3). Once metastatic spread has occurred, melanoma is highly lethal. Importantly, metastatic melanoma is highly resistant to chemotherapy and radiation therapy (4). In contrast, melanoma has been shown to be susceptible to immune-based therapeutic approaches (5–7). Thus, it is critical to further understand how melanoma evades immune-mediated clearance and how stromal and environmental factors influence melanoma immune surveillance.

Abbreviations: CPAF, carbamoyl PAF; CHS, contact hypersensitivity; DNFβ, dinitrofluorobenzene; EGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; mRNA, messenger RNA; NAC, N-acetyl cysteine; Ox-GPC, oxidatively modified glycerophosphocholine; PAF, platelet-activating factor; PAF-R, PAF-receptor; PBS, phosphate-buffered saline; qRT–PCR, quantitative reverse transcription–PCR; ROS, reactive oxygen species; Tregs, regulatory T cells; UVB, ultraviolet B radiation; WT, wild-type.

Exposure of human skin to the ubiquitous environmental stimulus ultraviolet B radiation (UVB, 290–320 nm) is associated with both acute and chronic consequences ranging from the induction of inflammatory responses to immunosuppression and development of malignant melanoma and non-melanoma skin cancer (8–10). It is becoming recognized that UVB-initiated effects are mediated in part through membrane phospholipids. UVB irradiation is a potent pro-oxidative stressor (11,12) and can induce reactive oxygen species (ROS), generating lipid mediators as a direct result of lipid peroxidation. Among these biologically active oxidized lipids, the proinflammatory phospholipid platelet-activating factor (PAF; 1-O-alkyl-2-acetyl glycerophosphocholine) can be produced by UVB radiation through non-enzymatic-free radical attack on the sn-2 fatty acid of parent glycerophosphocholines as well as via tightly controlled enzymatic pathways (12). In addition to native PAF, this sn-2 oxidation also results in the production of other oxidized glycerophospholines (Ox-GPCs) exhibiting PAF-like activity (13). Once produced, PAF stimulates diverse biological functions such as acute inflammatory responses (e.g. anaphylaxis) and delayed processes such as systemic immunosuppression (14–17). PAF agonist production via ROS contrasts with the tightly controlled enzymatic pathways of PAF synthesis (18,19).

PAF and PAF-like species exert their effects via binding to a single specific transmembrane G-protein-coupled receptor, the PAF-receptor (PAF-R) (19). The expression of the PAF-R has been identified in immune cell types as well as keratinocytes of the skin (20,21). The interaction of PAF with PAF-R has been shown to activate various intracellular signaling pathways including intracellular calcium ion mobilization, phosphatidylinositol turnover and activation of cellular phospholipases and intracellular kinases (22,23).

Several lines of evidence have implicated the PAF system in melanoma pathogenesis. First, though not expressed on primary melanocytes, PAF-Rs have been reported to be expressed on melanoma cell lines (24). Second, several studies have demonstrated that PAF directly stimulates melanoma cell growth (25–27). Injection of B16F10 murine melanomas together with PAF in matrigel results in an augmentation of tumor growth in vivo (25). Overexpression of the PAF catabolizing enzyme, PAF-acetylhydrolase, in melanoma tumor cells has been reported to decrease tumor growth in vivo (26). Finally, systemic treatment with the PAF-R antagonist WEB2170 also inhibits experimental melanoma tumor growth (27). What is not clear from these studies, however, is whether host/stromal PAF-R or tumor cell PAF-R is mediating these effects.

UVB-mediated systemic immunosuppression has been classically measured by the ability of distant UVB irradiation to inhibit contact hypersensitivity (CHS) responses to chemical antigens such as dinitrofluorobenzene or delayed type hypersensitivity responses to antigens such as Candida (15). In regards to cell types and mediators involved, studies from several laboratories have demonstrated the importance of ROS, PAF, IL-10, cyclooxygenase-2 and mast cells (12,15–17,28). UVB has been shown to induce systemic immunosuppression in humans (29). Though UVB-mediated systemic immunosuppression has been linked to photocarcinogenesis, the significance of UVB-mediated systemic immunosuppression in other processes including melanoma progression is unknown (30). The present studies were designed to test whether systemic immunosuppression induced by UVB can modulate tumor growth in a murine model of melanoma tumor progression. This investigation demonstrates that UVB irradiation augments melanoma tumor growth via the generation of PAF-R agonists that act upon the host in a process that involves IL-10 and regulatory T cells (Treg). Delineation of the ability of environmental pro-oxidative stressors such as UVB to modulate melanoma tumor growth could have significant clinical implications in terms of...
Ox-GPC PAF agonists inhibit antitumor immunity

Materials and methods

Reagents and UVB irradiation source
All chemicals were obtained from Sigma–Aldrich (St Louis, MO) unless indicated otherwise. RPMI 1640 media was ordered from Mediatech (Manassas, VA) and G418. Penicillin and streptomycin were from Invitrogen (Frederick, MD). Antibody against mouse anti-IL-10 was purchased from BD Biosciences (San Jose, CA), and anti-CD25 was from BioXcell (West Lebanon, NH). Isotype control antibodies against IgG1 and IgM were obtained from e Biosciences (San Diego, CA) and BioXcell. Marine PAF-R, enhanced green fluorescent protein (EGFP), CD3 and glyceraldehyde 3-phosphate dehydrogenase RT primers, first-strand synthesis and SYBR Green quantitative reverse transcription–PCR (qRT-PCR) reagents were purchased from SuperArray Bioscience Corp (Frederick, MD). As previously reported (16,17) UV source was a Philips F20T12/UVB lamp. The intensity of the UVB source was measured before each experiment using an IL1700 radiometer and a SED240 UVB detector (International Light) at a distance of 8 cm from the UVB source to the anesthetized mice.

Cells and mice
The mouse melanoma B16F10 cell line, originally obtained from a metastatic lesion from the lungs of C57BL/6 mice (31), was maintained in RPMI 1640 (Mediatech) supplemented with 10% fetal bovine serum (Invitrogen) and 100 μg/ml penicillin and streptomycin (Invitrogen). A B16F10-PAF-R model system stably expressing the PAF-R was created by transduction of PAF-R-negative B16F10 cells with the MSCV2.1 retrovirus encoding the human leukocyte PAF-Receptor as described previously (32). B16F10 cells stably transduced with the PAF-R receptor (designated as B16-PAF-R cells) or with control empty vector MSCV2.1 retrovirus (defined as B16-MSCV cells) were characterized by calcium mobilization studies using the calcium-sensitive dye Fura-2 (32) and by real-time quantitative PCR (16).

For in vivo experiments, a C57BL/6 background, generated as described previously (33), were a kind gift of Prof. Takao Shimizu (University of Tokyo Department of Biochemistry). C57BL/6-wild-type (WT) mice (PAF-R expressing; age 8–12 weeks) were purchased from the Charles River Laboratories. Immuno-deficient NOD.CB17-PrkdcSCID/J (common name: NOD SCID) mice were purchased from the Indiana University Simon Cancer Center Core facility. Foxp3EGFP knock-in transgenic mice on the C57BL/6 background (age 8–12 weeks) were procured from the Jackson Laboratories (34). These 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.

Measurement of intracellular calcium ion influx
The presence of a functional PAF-R in B16-PAF-R was identified by the ability of the metabolically stable PAF-R agonist, carbamoyl PAF (CPAF), to induce an intracellular Ca2+ mobilization response in B16-PAF-R cells as described previously (17). In brief, B16-PAF-R and B16-MSCV cells were preloaded with the Ca2+-sensitive indicator, fura-2-AM (4 μM in Hanks’ balanced salt solution) at 37°C for 90 min, washed and resuspended in Hanks’ balanced salt solution at room temperature before use. CPAF dissolved in ethanol (adjusted to 1 μM) was added to an aliquot of these cells (1.0–1.5 × 106 cells/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 Ca2+ influx in suspensions was calculated as described (17) and shown as percentage of maximal peak calcium flux induced by 1 μM CPAF.

In vivo tumor growth studies
To determine the effect of UVB-induced systemic immunosuppression in melanoma growth, 0.5 × 106 B16F10 cells, which lack functional PAF-R, were implanted s.c. into the shaved right hind flank of WT and Foxp3EGFP knock-in transgenic mice on the C57BL/6 background (age 8–12 weeks) and divided into two groups (sham versus UVB; 6 mice per group) and 0.5 × 106 B16F10 cells were implanted followed by treatment with either sham or UVB as mentioned above. To delineate whether host cell PAF-R or melanoma tumor cell PAF-R is playing a significant role in CPAF-induced enhanced melanoma tumor growth, 0.5 × 106 PAF-R-expressing (B16-PAF-R) or its vector control (B16-MSCV) cells were implanted into WT mice (six to nine mice per group) and treated with vehicle (50 μl phosphate-buffered saline (PBS)) or with 250 μg of CPAF (diluted in 50 μl PBS) on day 0, 6 and 12. To further confirm the role of host PAF-R, similar experiments were performed in Foxp3−/− mice (6–7 mice per group).

To evaluate the involvement of host immune cells in CPAF-mediated systemic immunosuppression and the resulting increase in melanoma tumor growth, 0.5 × 106 B16F10 cells were implanted into immunodeficient NOD SCID mice (eight mice/group) followed by treatment with either vehicle or CPAF as mentioned above.

To evaluate the involvement of IL-10 in UVB-induced augmentation of melanoma tumor growth and to determine whether the CPAF agonist CPAF mimics UVB-induced effects, WT mice (six mice per group) were first implanted with 0.5 × 106 B16F10 cells and then injected either with depleting antibody against mouse IL-10 or isotype control (IgG) antibody (100 μg per mouse) i.p. These mice were then treated with CPAF (250 μg), p. or UVB irradiated (5000 J/m2) on days 0, 6 and 12. For control groups, WT mice were either injected with vehicle (50 μl PBS) or left unirradiated (sham).

To determine the role of Tregs in UVB-induced melanoma tumor progression, Foxp3EGFP knock-in transgenic mice on the C57BL/6 background were injected with either depleting antibodies against CD25 (clone PC6.5.3 against IgG1 and 7D4 against IgM1) or isotype controls (IgG1 and IgM1) (1 mg each) 2 days before (−2 days) UVB irradiation. After 2 days, 0.5 × 106 B16F10 tumor cells were implanted followed by either sham or UVB irradiation as described above. The depletion of CD4+ CD25+ Tregs in Foxp3EGFP mice was confirmed (on day 10; including 2 days of either isotype control or anti-CD25 antibody injection) by analyzing the expression of EGFP as a surrogate for Tregs in inguinal lymph nodes and spleen by flow cytometry analysis in a separate experiment.

In all the above experiments, tumor growth (length and width) was monitored and measured daily until day 14 with digital calipers, and tumor volume was calculated (length/width2). At the end of the experiment (day 14), mice were euthanized by CO2 asphyxiation and cervical dislocation; tumors were excised and measured. In addition, tumors were processed for histology, stored in RNA later for real-time quantitative PCR analysis and fluorescence-activated cell sorting (FACS) analysis.

Real-time RT–PCR
Total RNA was extracted from melanoma tissues using the RNAeasy kit (QIAGEN). In brief, tissue was homogenized in a RLT buffer containing 1% (v/v) β-mercaptoethanol by bullet bead lysis (Next Advance, NY) using bead carnivores following the manual’s protocol (QIAGEN). Purified RNA was quantitated with the Nano Drop 2000 (Thermo Fisher Scientific, Lafayette, CO). Reverse transcription of whole RNA was done using Super Script cDNA synthesis kit (Invitrogen) with random hexamers. Complementary DNA was analyzed for content using an SYBR green-based, quantitative, reverse transcription PCR method (SA Biosciences, Frederick, MD) for PAF-R and EGFP and normalized against glyceraldehyde 3-phosphate dehydrogenase and CD3 as endogenous controls. Each assay was performed in duplicate in a 20 μl reaction volume with 2 × SYBR green master mix 2 μl of complementary DNA and primers at 10 μM. The following PCR conditions were used: 95°C for 15 min, followed by 45 cycles of 94°C for 15 s, 50°C for 30 s and 72°C for 30 s, followed by 72°C for a final 10 min. Each PCR reaction was also tested to assure a single product of the predicted size. Fluorescence was detected with the Step One Real-time PCR machine (Bio-Rad Laboratories, Hercules, CA). Quantification of each PCR product was expressed relative to glyceraldehyde 3-phosphate dehydrogenase or CD3.

Flow cytometry analysis
Tissue samples (draining lymph nodes and spleens) were harvested from isotype control antibody or anti-CD25 antibody treated Foxp3EGFP mice at day 10 (including −2 days of antibodies injection) and processed for the flow analysis. In brief, these tissues were dissociated into single cell suspension in RPMI media containing 10% fetal bovine serum using Gentle MACS TM strainer (40 μm) and centrifuged at 12 000 r.p.m. for 5 min. Spleens were processed initially with red blood cell lysis buffer. Cells from each tissue sample were washed twice with FACS buffer (PBS containing 2% fetal bovine serum) and counted. 1 × 106 cells from each organ were incubated with Fc blocking (CD16/32) antibody for 10 min after which cells were washed twice with FACS buffer. Foxp3-positive cell populations were

1361
quantitated based on EGFP as surrogate by FACS. Data files were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis
In the present study, at least five mice per group were used in all murine experiments. Statistical analyses were conducted using SAS version 9.3 (Cary, NC). Shapiro–Wilk’s and Levene’s tests were used to test normality and equal variance for days 12 and 14 tumor volume, tumor weight and qRT–PCR values. Normality was a reasonable assumption in all cases. For comparing two groups, we used equal or unequal variance t-tests. For comparing more than two groups, we used analysis of variance (with the Welch approximation if the variances were unequal) and post hoc Tukey-adjusted pair-wise tests. Only the P values from the overall F-tests are presented in the text. Statistical significance was defined as a P value <0.05.

Results
UVB-induced augmentation of melanoma tumor growth is dependent on the PAF-R system
Previous studies have demonstrated the importance of PAF-R signaling in UVB-mediated systemic immunosuppression as measured by the inhibition of CHS reactions to the contact allergen dinitrofluorobenzene (DNFB) using C57BL/6 mice expressing PAF-R (WT) and PAF-R-deficient mice (Pafr−/−) (16,17). Our first studies tested whether UVB irradiation at doses 2500, 5000 and 7500 J/m², which induce systemic immunosuppression, could augment experimental murine melanoma growth. The dose of UVB irradiation (5000 J/m²) was selected by performing a pilot study in WT mice (five mice per group) harboring B16F10 cells followed by either sham or UVB irradiation at doses 2500, 5000 and 7500 J/m² (Supplementary Figure 1 is available at Carcinogenesis Online and data not shown). The dorsal skin of WT and Pafr−/− mice was shaved, and 5 × 10⁵ B16F10 cells were inoculated s.c. into the right flank of each mouse. This was followed by UVB irradiation (5000 J/m² on day 0, 6 and 12) on an ~2.5 × 2.5 cm area of shaved dorsal skin which was ~3 cm away from the shielded tumor injection site (Figure 1A). Tumor growth was measured daily with calipers. As shown in Figure 1B, C and D, UVB irradiation of WT mice augmented the growth of B16F10 mouse melanoma tumors significantly compared with their non-irradiated (sham) counterparts. Importantly, Pafr−/− mice were resistant to this UVB-mediated augmentation of melanoma tumor growth (Figure 1B and D).

Effect of antioxidants on UVB-induced melanoma tumor progression
Our previous studies have demonstrated that UVB irradiation resulted in the production of PAF and PAF-like Ox-GPCs via its ability to induce ROS (12,13). Feeding WT mice a vitamin C-enriched diet (10 g/kg) and 5 mM NAC in water for 10 days followed by the treatment with UVB as outlined in Materials and methods. As shown in Figure 2, the UVB-mediated increase in B16F10 melanoma tumor growth was significantly attenuated in the group of mice, which were treated with vitamin C/NAC as compared with UVB-treated mice on a regular diet. Final tumor weights mirrored the tumor volume measurements, demonstrating increased weight of tumors from UVB-irradiated hosts fed normal diets compared with those fed with an antioxidant diet (data not shown). Of note, other studies demonstrated that systemic administration of vitamin C or NAC alone (five mice per group) exerted only partial protection against UVB-induced melanoma tumor progression (Supplementary Figure 2 is available at Carcinogenesis Online). The finding that antioxidants vitamin C and NAC could block UVB-mediated augmentation of tumor growth suggests the involvement of oxidatively produced PAF species rather than enzymatically produced ones.

Importance of host PAF-R signaling in melanoma tumor progression
Since PAF-Rs have been reported to be expressed on melanoma cells (24) and PAF-R activation has been reported to enhance tumor growth (25–27), the next studies were designed to define whether the augmentation of tumor growth by PAF-R signaling was due to direct effects of PAF on either the tumor or on the host cells. We first tested B16F10 cells for PAF-R messenger RNA (mRNA) expression by RT–PCR and for PAF-R activity by functional intracellular calcium ion mobilization studies using calcium-sensitive FURA-2 dye. These studies did not find significant levels of PAF-R transcripts in B16F10 cells nor did B16F10 cells respond with a calcium mobilization response to the PAF-R agonist, CPAF (data not shown). To test the role of the melanoma PAF-R in tumor cell growth, we transduced B16F10 cells with a PAF-R or empty MSCV2.1 retroviral construct to create B16F10PAF-R (B16-PAF-R) and B16F10MSCV2.1 (B16-MSCV), respectively. PAF-R mRNA expression was present in B16-PAF-R cells as demonstrated by qRT–PCR (Supplementary Figure 3 is available at Carcinogenesis Online), and PAF-R function was confirmed by intracellular calcium mobilization studies in response to CPAF (Figure 3A and B). Using these cell lines, we next assessed whether or not PAF-R expression in B16F10 cells alone would exert a growth promoting effect on melanoma tumors. To that end, 5 × 10⁶ B16-PAF-R or B16-MSCV cells were implanted into the flanks of WT mice (six mice per group) and the growth of melanoma tumors was recorded. We observed that the expression of PAF-R alone was not associated with increased melanoma tumor growth as no difference in the tumor volumes or tumor weights was seen with these cells when implanted into WT mice (Supplementary Figure 4 is available at Carcinogenesis Online). We then evaluated the effect of PAF-R activation on the tumor growth of B16-PAF-R and B16-MSCV cells. To that end, we implanted 5 × 10⁶ B16-PAF-R or B16-MSCV cells into the flanks of WT mice followed by treatment with or without PAF-R agonist (CPAF; 250 ng per mouse on day 0, 6 and 12). This dose of CPAF was used as our previous studies have demonstrated that i. p. injection of 250 ng CPAF mimics UVB-induced inhibition of CHS reactions to DNFB (17). As shown in Figure 3C and D, CPAF treatment resulted in a significant increase in tumor growth regardless of the PAF-R status of the B16F10 cells (no significant differences were noted between the tumor growths of B16-MSCV and B16-PAF-R cells exposed to CPAF treatment at day 14), suggesting the role of host PAF-R signaling rather than melanoma tumor cell PAF-R in mediating CPAF-induced melanoma tumor growth.

To further confirm the role of the host PAF-R signaling in mediating melanoma tumor growth, we performed the same studies in Pafr−/− mice, which demonstrated that CPAF failed to augment the growth of B16F10 cells regardless of the status of their PAF-R expression (Figure 3E and F). These studies indicate that activation of the PAF system augments the growth of B16 tumors in a manner dependent on host cell expression, but not melanoma tumor cell expression, of the PAF-R.

Role of immune cells in mediating PAF-R effects on melanoma tumor growth
The next studies were designed to assess the importance of the adaptive immune system in the PAF-R-mediated augmentation in an experimental murine melanoma tumor growth model. To that end, the ability of systemic CPAF to modulate tumor growth in T- and B-cell deficient severe combined immunodeficient (NOD SCID) mice was tested. As depicted in Figure 4A and B, CPAF treatment had no appreciable effect on tumor growth in NOD SCID mice. Of note, B16F10 tumors grew approximately twice as fast in these immunodeficient mice in comparison with their WT counterparts. In fact, the rate of tumor growth of B16F10 cells in NOD SCID mice resembled WT mice treated with UVB or CPAF. These findings demonstrate the importance of the adaptive immune system in mediating PAF agonist-mediated augmentation of melanoma tumor growth and reflect the immunosuppressive effect of UVB and CPAF.
Involvement of IL-10 in UVB/CPAF-induced melanoma tumor progression

The following lines of evidence indicate the immunomodulatory cytokine IL-10 mediates PAF-induced immunosuppression. First, activation of the PAF-R in the murine epithelial cell line PAM-212 (15) as well as i.d. CPAF injection induce epidermal production of IL-10 in PAF-R-expressing WT mice (16). Second, UVB irradiation increases IL-10 mRNA levels in the lymph nodes and spleens of WT mice as compared with their respective control groups (16). Finally, depleting antibodies against IL-10 block both CPAF- and UVB-induced inhibition of CHS reactions to DNFB in WT mice (16).

The following studies were designed to assess whether IL-10 is involved in UVB or CPAF-induced melanoma tumor progression.

WT mice engrafted with B16F10 tumor cells were injected with either depleting antibodies against IL-10 or isotype control antibodies, followed by the treatment with either CPAF or UVB and monitoring tumor growth. As shown in Figure 5, anti-IL-10 antibodies blocked both CPAF- and UVB-mediated melanoma tumor progression compared with isotype control antibody injected CPAF or UVB-treated mice. These studies indicate that IL-10 mediates both CPAF- and UVB-induced melanoma tumor progression.

Role of regulatory T cells in UVB-induced melanoma tumor progression

Recently, CD4<sup>+</sup>CD25<sup>+</sup> Tregs have been shown to mediate UVR-induced localized immunosuppression in a process involving
that UVB-induced generation of ox-GPCs having PAF-R agonistic activity has been demonstrated, it is not known whether the systemic PAF system in mediating UVB-induced augmentation of melanoma tumor growth: C57BL/6 (WT) mice were either fed vitamin C-enriched chow diet and 5 mM NAC in water for 10 days or standard diet/water. The mice then underwent the protocol of UVB irradiation and melanoma implantation outlined in Figure 1. The tumors were measured daily with calipers. The data are represented as mean ± SE of tumor volume from six to seven mice each group. Significant differences were observed (day 12, $P$ value <0.01; day 14, $P$ value <0.0001) with the regular diet + UVB group being different from the two vitamin C groups on day 12 and being different from all other groups on day 14.

**Fig. 2.** Treatment with vitamin C + NAC attenuates UVB-mediated augmentation of B16F10 melanoma tumor growth: C57BL/6 (WT) mice were either fed vitamin C-enriched chow diet and 5 mM NAC in water for 10 days or standard diet/water. The mice then underwent the protocol of UVB irradiation and melanoma implantation outlined in Figure 1. The tumors were measured daily with calipers. The data are represented as mean ± SE of tumor volume from six to seven mice each group. Significant differences were observed (day 12, $P$ value <0.01; day 14, $P$ value <0.0001) with the regular diet + UVB group being different from the two vitamin C groups on day 12 and being different from all other groups on day 14.

**Fig. 3.** Studies have implicated the involvement of CD4+$\text{CD}^{25^{+}}$-expressing regulatory T cell (Treg) in mediating UVR-induced immunosuppression. The ability of depleting antibodies against IL-10 and Tregs to block PAF-R-mediated augmentation of tumor growth also attests to the involvement of the adaptive immune response. Nevertheless, it is possible that systemic PAF-R agonists could contribute to melanoma growth via both effects on tumor immunity as well as direct effects on melanoma tumor growth. The ability of depleting antibodies against IL-10 and Tregs to block PAF-R-mediated augmentation of tumor growth also attests to the involvement of the adaptive immune response.

**Fig. 4.** Studies have implicated the involvement of CD4+$\text{CD}^{25^{+}}$-expressing regulatory T cell (Treg) in mediating UVR-induced immunosuppression. The ability of depleting antibodies against IL-10 and Tregs to block PAF-R-mediated augmentation of tumor growth also attests to the involvement of the adaptive immune response. Nevertheless, it is possible that systemic PAF-R agonists could contribute to melanoma growth via both effects on tumor immunity as well as direct effects on PAF-R-expressing melanoma tumors.

**Discussion**

Although the role of the PAF system in mediating UVB-induced systemic immunosuppression (14–17) and photocarcinogenesis (14,36) has been demonstrated, it is not known whether the systemic immunosuppressive effects of UVB could modulate melanoma tumor immunity. The present study lends support to the concept that UVB-induced generation of ox-GPCs having PAF-R agonistic activity augments the growth of experimental murine melanoma tumors. Moreover, these studies indicate that IL-10 and Tregs play critical roles in this PAF-R-dependent enhanced melanoma tumor progression.

The current study demonstrates that UVB irradiation to an area of skin distant from s.c. implanted B16F10 murine melanoma tumor augments tumor growth in PAF-R-expressing C57BL/6 (WT) mice compared with non-irradiated (sham) mice. The fact that UVB did not exert these growth-promoting effects in Pafr$^{-/-}$ mice and the ability of exogenous CPAF to mimic this effect, both confirm that the PAF system mediates the UVB effect. These results are consistent with previous reports demonstrating that UVB irradiation of murine and human skin generates novel lipid mediators (Ox[Black and white]-GPCs) having PAF-R agonistic activity and that these PAF-R agonists mediate UVB-induced systemic immunosuppression (12,13,15–17,37). Furthermore, the ability of antioxidants to block the UVB-mediated growth enhancing effects is evident that Ox-GPC PAF-R agonists are produced non-enzymatically in this process. Of note, pro-oxidative stressors other than UVB such as tert-butyl hydroperoxide and more clinically relevant agents such as chemotherapeutic drugs and cigarette smoke have also been reported to generate Ox-GPCs having PAF-R agonistic activity (38–40). Thus, it is possible that exposure to environmental pro-oxidative stressors other than UVB could generate this pro-tumor growth effect (39,40). This process could in turn explain the beneficial effects reported for combining systemic antioxidants with chemotherapeutic agents for treating human malignancies (41,42).

Previous studies have demonstrated that PAF-R agonists augment melanoma tumor growth in vitro and in vivo, effects that have been proposed to be a direct effect on the tumor cell PAF-R (24–26). For example, Melnikova et al. (24) have demonstrated that the PAF-R agonist, CPAF, increased the in vitro growth of PAF-R-expressing human melanoma cells in a dose-dependent manner in a process involving metalloproteinase 2. Similarly, these authors found that CPAF treatment augmented human melanoma growth in SCID mice (24). In contrast, the present studies, through the use of murine PAF-R-negative and -positive tumor cells and immunocompetent hosts, indicate that PAF-R agonistic (CPAF) effects on the growth of s.c. implanted B16F10 melanoma tumor cells are due to host rather than direct tumor effects. In addition, implantation of only PAF-R-negative and -positive tumor cells into WT (PAF-R expressing) host without the addition of CPAF did not result in differences of tumor growth. Differences between the findings of previous studies indicating that PAF-R activation directly augments melanoma tumor growth could be due to differences in the signaling pathways induced by the native versus ectopically expressed PAF-R used in these studies.

The present studies demonstrating the lack of effect of exogenous CPAF on tumor growth in immunodeficient NOD SCID mice indicate that the adaptive immune system mediates this PAF-R-dependent response. The ability of depleting antibodies against IL-10 and Tregs to block PAF-R-mediated augmentation of tumor growth also attests to the involvement of the adaptive immune response. Nevertheless, it is possible that systemic PAF-R agonists could contribute to melanoma growth via both effects on tumor immunity as well as direct effects on PAF-R-expressing melanoma tumors.

Studies have implicated the involvement of CD4+$\text{CD}^{25^{+}}$-expressing regulatory T cell (Treg) in mediating UVB-induced immunosuppression (35,43–45). Tregs have also been recognized as playing an active role in the prevention of autoimmunity, graft versus host disease and transplant rejection (46). The development and function of a key population of Tregs are regulated by a member of the forkhead transcription factor family protein, FoxP3 (47). Several lines of evidence suggest the possible involvement of Tregs in mediating UVB-induced local as well as systemic immune tolerance. First, Elmets et al. (43) have observed that application of DNFB onto UV-irradiated skin results in the suppression of Tregs that suppress CHS. Second, UVB-induced systemic immunosuppression in mice is associated with an increase in the number of Tregs, which is suppressed by the treatment with prostaglandin E2 receptor subtype 4 (EP4) antagonist (44). Third, IL-10,
which is necessary for UVB-mediated systemic immunosuppression, is an important cytokine for Treg function (35,45). Finally, the Schwarz group has characterized the phenotypic and functional aspects of UV-induced Tregs in a local immunosuppression model and demonstrated that UVB irradiation activates Tregs that secrete IL-10 which promotes suppression of CHS (45). This UVB-mediated tolerance was blocked by i.p. injection of depleting antibody against IL-10 (45). These separate lines of evidence all suggest that Tregs play an important role in UVB-mediated immunosuppression. In addition to Tregs, IL-10-producing regulatory B cells, which are generated via UV radiation, could be involved in this process (48).

The present studies demonstrate that Tregs play an important role in Ox-GPC-induced melanoma tumor growth as depleting antibodies against CD25 substantially block UVB-induced tumor growth in FoxP3EGFP mice. Furthermore, the expression of EGFP mRNA, a marker for FoxP3 expressing Tregs, was significantly increased in the tumors of UVB-irradiated mice, an effect that was lost in mice treated with depletion of CD25+ cells. Consistent with these findings, selective depletion of FoxP3-expressing Tregs has been shown to improve vaccination strategies against established melanoma (49,50).

Of interest, Donawho et al. (51) reported 20 years ago that injection of murine melanoma and other tumors into UV-irradiated skin resulted in an augmentation of tumor growth. However, UV irradiation of one ear did not augment tumor growth in the other ear, suggesting that this was a local effect. Given that there appears to be a critical threshold for the amount of UVB to allow adequate production of Ox-GPC PAF-R agonists to augment melanoma tumor growth (Supplementary Figure 1 is available at Carcinogenesis Online), it is possible that the lack of efficacy of systemic UV previously reported could be due to not enough PAF-R agonists produced in their system.

In summary, the present studies indicate that UVB-generated Ox-GPCs with PAF-R agonistic activity target host adaptive immune cells, most notably Tregs, to mediate systemic immunosuppression thus enhancing experimental murine melanoma tumor growth. These data also suggest that host expression of PAF-R, but not melanoma
tumor cell expression of PAF-R, is involved in UVB/PAF-R-induced augmentation of melanoma tumor growth. These studies describe a previously unrecognized mechanism by which an environmental stressor allows melanoma tumor cells to escape antitumor immunity. Inasmuch as other pro-oxidative stressors could potentially generate PAF-R agonists, this pathway could have clinical relevance.

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Ox-GPC PAF agonists inhibit antitumor immunity

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1367