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Xeroderma Pigmentosum A Deficiency Results in Increased Generation of Microvesicle Particles in Response to Ultraviolet B Radiation

Christian Lea Rajeshkumar
Wright State University

XERODERMA PIGMENTOSUM A DEFICIENCY RESULTS IN INCREASED GENERATION OF MICROVESICLE PARTICLES IN RESPONSE TO ULTRAVIOLET B RADIATION

A Thesis submitted in partial fulfillment of the
requirements for the degree of
Master of Science

by

LEA RAJESHKUMAR CHRISTIAN

B. Pharm., Gujarat Technological University, India, 2019

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WRIGHT STATE UNIVERSITY

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Lea Rajeshkumar Christian ENTITLED Xeroderma Pigmentosum A Deficiency Results In Increased Generation Of Microvesicle Particles In Response To Ultraviolet B Radiation BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Jeffrey B. Travers, M.D., Ph.D.
Thesis Director

_____
Jeffrey B. Travers, M.D., Ph.D.
Chair, Department of
Pharmacology and Toxicology

Committee on Final Examination:

_____
Jeffrey B. Travers, M.D., Ph.D.

_____
Michael G. Kemp, Ph.D.

_____
Ji Chen Bihl, M.D., Ph.D.

_____
Barry Milligan, Ph.D.
Vice Provost for Academic Affairs
Dean of the Graduate School

ABSTRACT

Christian, Lea Rajeshkumar, M.S., Department of Pharmacology and Toxicology, Wright State University, 2021. Xeroderma Pigmentosum A Deficiency Results in Increased Generation of Microvesicle Particles in Response to Ultraviolet B Radiation.

Xeroderma Pigmentosum is a genetic disorder in which ability to repair DNA damage such as from UV radiation is decreased. Nucleotide excision repair is known for repairing DNA damage caused by UV radiation and *XPA* plays a major role in recognizing and eliminating abnormal section of DNA. Therefore, *XPA* deficiency decreases repair efficiency of DNA. Of note, *XPA* deficiency is linked with photosensitivity. Microvesicle particles are membrane-bound particles which are released into the extracellular environment in response to multiple stimuli including the lipid Platelet activating factor (PAF). Previous studies have shown that *XPA* deficiency can induce increase production of reactive oxygen species and generates large amounts of PAF agonists produced non-enzymatically. Hence, the present studies are designed to study if *XPA* deficiency induces higher UVB-MVP release via PAF-R signaling pathway. Studies involving a *XPA*- deficient keratinocyte cell-line were able to show that UVB irradiation can cause increase MVP release. Similarly, *XPA* knockout (KO) mice generated increased MVP with UVB irradiation both in skin as well as plasma in comparison to wild-type mice. Increased production of cytokines (TNF-alpha and IL-6) were also seen in *XPA* KO mice. However, absence of *XPA* did not affect MVP release when treated with PAF-R agonist or phorbol ester TPA. Topical application of the acid sphingomyelinase (aSMase) inhibitor imipramine was able to inhibit UVB induced MVP release and pro-inflammatory cytokines. Likewise, genetically knocking down aSMase affected MVP release by UVB irradiation in comparison to wild-type and *XPA* KO mice. As MVP been involved in UVB signaling, inhibiting MVP release by pharmacological means might be a novel therapeutic approach in photosensitive conditions.

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ABBREVIATIONS

XPA – Xeroderma Pigmentosum A

WT – Wild-type

aSMase – Acid Sphingomyelinase

MVP – Microvesicle Particles

PAF-R – Platelet activating factor receptor

ROS – Reactive oxygen species

HaCaT – Heat and Calcium Transformed keratinocyte cell line

NER – Nucleotide Excision Repair

KO – Knockout

HBSS – Hanks' Balanced Salt Solution

UVB – Ultraviolet B Radiation

PAF – Platelet activating factor

DMEM – Dulbecco's Modified Eagle Medium

CPAF – Carbamoyl PAF

TPA – Tetradecanoyl phorbol acetate

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During my thesis research I have received a great deal of support and encouragement. I would first like to thank my thesis advisor Dr. Jeffrey Travers; whose expertise was irreplaceable in either creating methodology or finding an alternative approach. I very much appreciate his constructive guidance that has made a great difference in my research. I would also like to thank my thesis committee members Dr. Michael Kemp and Dr. Ji Bihl for their unparalleled knowledge and providing assistance while learning new techniques. I would also like to extend my greatest gratitude to Ms. Christine Rapp for her constant guidance and valuable advice that has made a huge impact in my thesis research. I am extremely grateful of Dr. Christina Borchers and Dr. Langni Liu for their invaluable intelligence. I gratefully acknowledge the help I received from Dr. Christina Borchers. I also had great pleasure working with my colleagues Shweta Bhadri, Arikatla Venkata Sravya and Chad Brewer. A genuine appreciation also goes to Dr. Chen's Lab, Dr. David Cool's Lab, Dr. Anita Thyagarajan, Dr. Ravi Sahu and the entire Pharmacology and Toxicology Department, Wright State University. Last but certainly not the least, I would like to thank my parents for their wise counsel and support.

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DEDICATION

This work is dedicated to my parents Mr. Rajeshkumar Christian and Mrs. Hemerline Christian. Thank you for your endless love and encouragement!

CHAPTER I – INTRODUCTION

STATEMENT OF PROBLEM

Photosensitivity is a condition that could damage skin and cause skin cancer. It has also shown to have a major role in pathophysiology of Lupus Erythematosus. Although a lot of mechanisms and pathways have shown to contribute to severe photosensitive conditions, therapeutic strategies which could be useful in photosensitivity have not yet been determined. We believe that MVPs generated by activation of ROS and PAF-R agonists is involved in causing severe "photosensitive" responses such as redness, cytokines release and that these responses might be inhibited by blocking acid sphingomyelinase.

SIGNIFICANCE OF RESEARCH

If enhanced MVPs are released by the lipid platelet activating factor pathway in Xeroderma pigmentosum A deficient model then approaches that could block MVPs release would be considered very useful in photosensitive conditions.

STATEMENT OF PURPOSE

The purpose of this study is to know if enhanced reactive oxygen species and activation of PAF-R in *XPA* deficient models produce increased microvesicle particles by UV radiation that can cause "photosensitive" response and by blocking aSMase can reduce these responses in *XPA* deficient model.

LITERATURE

XERODERMA PIGMENTOSUM

Xeroderma Pigmentosum (*XP*) is a rare autosomal recessive condition (Kunisada, 2017). *XP* was first described by a physician through reporting a patient with dry skin showing wrinkles and tremendous sensitivity to developing skin-based tumors (JO, 2016). Later, the pathophysiology of the disease was recognized by intense sun sensitivity which induces high risk for skin tumors. *XPA* is a DNA Damage

Recognition and Repair Factor in Nucleotide Excision Repair pathway (NER) (Yao, 2012).

NER is involved in repairing DNA damage caused by Ultraviolet Radiation. NER pathway can be classified into two groups : Global Genome – Nucleotide Excision Repair (GG-NER) and Transcription – Coupled Nucleotide Excision Repair (TC-NER) (Marteijn, 2014). In the DNA damage system, GG-NER studies the whole genome for damage, UVR – DNA damage binding protein is involved in recognizing damage DNA while in TC-NER, DNA damage is indirectly recognized by the stalling of RNA Polymerase II (Marteijn, 2014). In NER, nine major proteins are involved and in that proteins which are responsible for causing disease are *XPA, XPB, XPC, XPD, XPE, XPF* and *XPG*. *XPA* has a significant function in NER pathway. *XPA* binds with damaged part of DNA and interacts with almost all proteins and these proteins forms a complex to unwind the part of damage DNA (Yao, 2012). Therefore, *XPA* deficiency leads to decrease efficiency in recognizing and repairing UVB damage in DNA and thus linked to photosensitivity. UV-induced autoimmune response has a pathogenic role in autoimmune disease such as Lupus Erythematosus (LE) (Lehmann P, 2009). Recent studies have demonstrated that photosensitivity is the most common indicator in representing LE (Lehmann P, 2009). Photosensitivity is considered as an early sign for lupus spreading systemically.

ULTRAVIOLET RADIATION

Sun, which is the natural source of Ultraviolet Radiation emits UVA, UVB and UVC. UVA penetrates deep into the dermis layer as it has the longest wavelength which is 320 nm – 400nm. UVB has wavelength of 290nm – 320nm and it reaches to epidermis layer of skin (Ullrich, 2005). UVC has wavelength of 100nm – 290nm, UVC rays are the shortest waves which usually do not infiltrate the ozone layer. Both UVA and UVB can cause skin damage, sunburn, aging and wrinkles. Even though, UVA comprises 95% of the UV radiation that reaches the earth surface, the role of UVB in causing non-melanoma skin cancer, the most common type of skin cancer and immunosuppression is well known (Ullrich, 2005) (Yao, 2012). To

initiate immunosuppression different photoreceptors are being identified that can absorb UV radiation, among them membrane lipid peroxidation and free radial formation, *trans*- urocanic acid and epidermal DNA are considered as major photoreceptors (Ullrich, 2005). In UV-induced immunosuppression, free radical formation might play an important role. UV exposure can trigger activation of Src tyrosine kinase. This leads to stimulation of downstream mediators such as Raf-1, c-jun amino terminal protein kinase (JNK) and finally phosphorylation of regulatory sites in the activation domain of c-jun leads to the NF-kB and AP-1 activation (Ullrich, 2005). Studies have shown that antioxidants treatment blocks the UV-induced free radical damage.

PLATELET ACTIVATING FACTOR

Platelet Activating Factor, also known as acetyl-glyceryl-ether-phosphorylcholine (AGEPC) or PAF, is a potent lipid mediator known to exert its effect in inflammation, platelet aggregation and sensitivity through G-protein coupled receptor, specifically called as platelet activating factor receptor (PAF-R) (Yao Y, 2009) . The PAF-receptor is known to expressed on variety of cells such as neutrophils, mast cells, keratinocytes, fibroblasts, platelets, eosinophils (Damiani E, 2016) (Yao Y, 2009). PAFR has many functions and a wide range of pathophysiological effects.

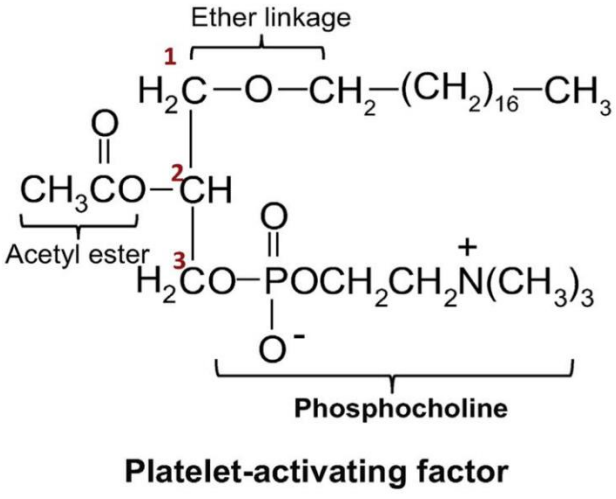


Figure 1: Structure of Platelet Activating Factor (Damiani E, 2016)

Structure and Chemistry of PAF

PAF is a 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine. PAF includes three structural features (Figure- 1) – 1. At *sn*-1 position, long chain alkyl group is present with an ether bond 2. At *sn*-2 position, formation of glycerol through esterification of acetic acid

3. At *sn*-3 position, phosphocholine group is present (Damiani E, 2016). Alkyl group at position 1, can have 12 to 18 carbon atoms in length, hence, signifies as family of phospholipids.

Synthesis of PAF

PAF is synthesized both enzymatically and non-enzymatically. Enzymatic synthesis includes both a remodeling and de-novo pathways. The remodeling pathway consists of removal of fatty acids by phospholipase A_2 (PLA_2), mainly cytosolic PLA_2 as it is calcium-dependent and selective for arachidonic acid (AA) and generates intermediate lyso-PC (LPC). Finally, PAF is produced through attachment of acetyl group by LPC-acetyltransferases (LPCAT). Generally, inflammatory cells use this pathway in response to cell-specific stimuli (Damiani E, 2016) (Shimizu, 2009). Cellular stimuli include induction of intracellular calcium mobilization which leads to production of PAF (Travers JB, 1996). The de-novo pathway consists of enzymes that can synthesize PAF from pre-existing neutral lipid by adding phosphocholine. Non-enzymatic synthesis of PAF involves attack of free radicals on the unsaturated fatty acids that can leads to oxidation process and rearranging the bonds (Konger RL, 2008). This rearrangement produces phospholipid reaction products that have potent PAFR activity. In comparison to enzymatic PAF production, the generation of oxidized glycerophosphocholines (Ox-GPC) is not controlled. Various factors might be responsible for generating Ox-GPC such as reduced amount of antioxidant defenses and amounts of ROS produced. Various environmental stressors have also shown to generate Ox-GPC and they are, Ultraviolet B Radiation, cigarette smoke, radiation therapy, chronic ethanol exposure (Marathe GK, 2005) (Sahu RP, 2013) (Yang L, 2010). Acute PAF effects include inflammation while, chronic PAF effects include immunosuppression (Walterscheid JP, 2002).

EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) were once considered as waste carriers, are now recognized as their ability to exchange components between cells and because of this property they might act as carriers for sending signals in normal homeostasis

or results in pathological developments (Van Niel G, 2018). EVs are heterogenous cell-derived structures. Different subtypes of EV are: Microvesicle particles (MVPs) which are also known as microparticles, have a size of 0.1~1µm, Exosomes, smaller than MVPs are further divided into Large exosomes (90~120nm) and Small exosomes (60~80nm). Recently, new EVs are being identified and classified according to their size, Exomeres (< 50nm), Oncosomes (1~10µm) and Migrasomes (0.5~3µm) (Chuo, 2018) (Figure-2). Exosomes are generated from the micro vesicular bodies (MVB), they have a role in cell-cell communication as they act as carrier of mRNAs and proteins. Oncosomes are known to be larger EV and are produced from cancer cells by the process called budding. Migrasomes and Exomeres biological function are yet to be identified (Chuo, 2018).

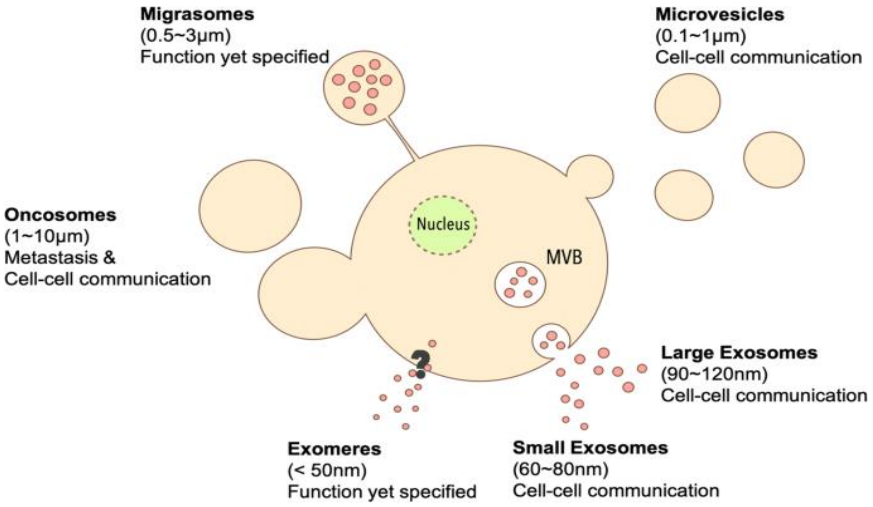


Figure 2: Schematic representation of different Extracellular vesicles differentiated by their size and secretion pathways. (Chuo, 2018)

Microvesicle Particles

Microvesicle particles (MVPs) (ectosomes or microparticle or microvesicle) are small membrane-bound particles which are released from the cell membrane via budding. Initially, known to be cell debris, MVPs have now been considered to have a role in keratinocyte cell signaling (Fahy K, 2017). The budding process through which MVPs are released into extracellular environment include different signaling pathways which involves upregulation of intracellular calcium

mobilization (Xiao X, 2015). Cellular and stimulus-dependent pathways are mainly involved in MVPs release. For example, in endothelial cells, MVPs are released due to p38 MAPK signaling (Norling, 2013) whereas, both p38 MAPK and Erk 1/2 pathways are involved in release of MVPs in THP-1 monocytes. Ceramides are also known to produced MVPs release. The cell membrane consists of lipids, cytokines, membrane receptors, adhesion molecules, enzymes and proteins and when MVP are generated, they carry membrane contents into extracellular space (Fahy K, 2017). Keratinocyte derived MVP have abundance of contents such as pro-inflammatory cytokines (IL-6, IL-8, TNF-alpha, IFN-gamma), chemoattractants (CXCL1, CXCL3, CXCL5 and CXCL6) and act as carrier of miRNAs (Shao S, 2020) (Kunisada, 2017). Because of these characteristics and functions, it is possible that MVP might a regulator of inflammation and immunosuppression.

Previous studies have shown that the environmental and oxidative stressor UVB can instigate production of reactive oxygen species (ROS) that can initiate the production of PAF agonists, ultimately, leads to activation of PAF-R through specific pathways. PAF-R activation, results in intracellular calcium mobilization, stimulation of mitogen activating protein kinase and activation of NF-kB pathway. Activation of PAF-R through abnormal production of ROS was also shown in photosensitive model (Yao, 2012). MVPs are generated because of various stimuli such as increase amount of intracellular calcium. The generation of MVPs by activation of PAF-R can be blocked by antioxidants specific inhibitors (Fahy K, 2017) (Bihl JC, 2016). These studies indicate that, MVP release might be an effector linking skin damage caused by environmental or oxidative stressor such as UVB involving activation of PAF system. If this is the case, then, pharmacologic or genetic approaches involving blocking of MVP release could be useful in conditions such as photosensitivity.

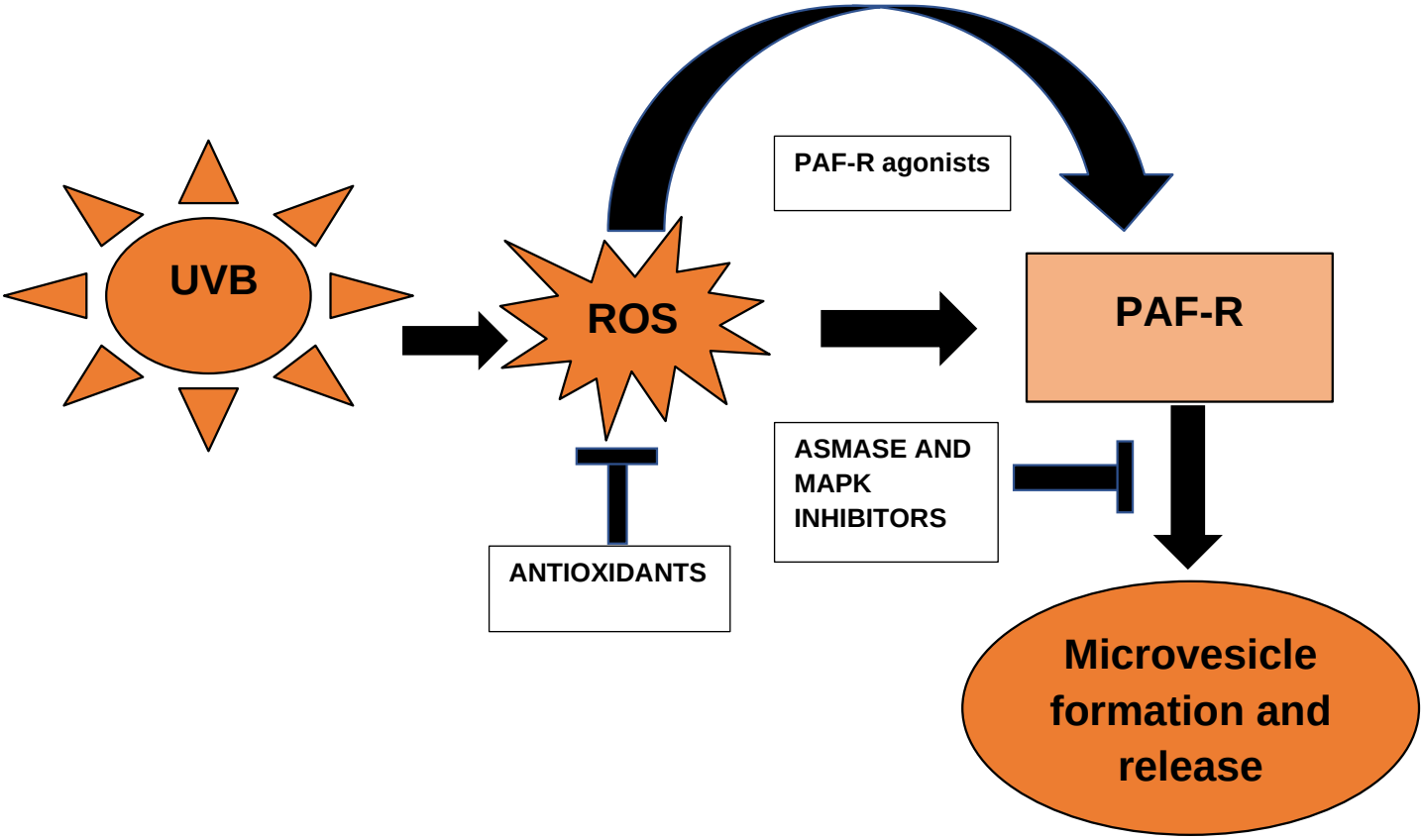


Figure 3: Model for UVB inducing Microvesicle via PAF-R signaling.

CHAPTER II - HYPOTHESIS AND SPECIFIC AIMS

HYPOTHESIS:

It is hypothesized that Xeroderma Pigmentosum A deficiency induces increased microvesicle particles release by Ultraviolet B Radiation via enhanced reactive oxygen species production and increased generation of platelet activating factor receptor agonists. Additionally, aSMase inhibitor reduces erythema, microvesicle particles release and cytokines production in the *XPA* photosensitive murine model.

SPECIFIC AIMS:

1. To compare UVB generated MVP in Human Keratinocytes cell line and murine models that lacks *XPA*, which have been previously shown to generate increase PAF-R agonists in response to UVB.
2. To determine the difference in MVP release by UVB that induces ROS in comparison to CPAF/TPA that does not induce ROS between group that lacks *XPA* and that which contain *XPA*.
3. To evaluate the role of aSMase and the effect of an aSMase inhibitor on UVB induced MVP release in photosensitive murine model.

CHAPTER III – MATERIALS AND METHODS

CELLS AND CELL CULTURE :

Cells were grown as previously described (Bihl JC, 2016) in 10 cm Petri dish using Dulbecco's Modified Eagle Medium(DMEM) with high glucose (GE Healthcare Life Sciences (Cytiva), HyClone, Cat No. SH30243.01) 10% FBS, 2mM glutamine, 100 U penicillin/ 0.1 mg/ml streptomycin, 0.25% Trypsin-EDTA (1X) (Gibco, Ref no. 25200-056), Phosphate Buffered Saline (FPBS) (1X) (PBS) 0.1 µM Sterile Filtered (Cytiva, HyClone Laboratories, Cat. No. SH30256.01), Hank's Balanced Salt Solution and 10mg/ml Bovine serum albumin (HBSS+BSA).

Human keratinocyte- derived cell line (HaCaT) and *XPA* deficient HaCaT cells (Kemp, 2016) were used in these studies. HaCaT cells, shows characteristics of primary cultured skin keratinocytes *in vitro* were grown in DMEM with high glucose under 5% CO_2 and 19% O_2 atmosphere, temperature 37°C. Fresh medium was added every 2- 3 days. With cell confluency of 80 to 90%, DMEM was discarded and washed with FPBS twice and trypsinized the cells with 2ml of 0.25% trypsin-EDTA and kept in incubator for 10 - 15 minutes.

In the 10 cm plate 8 ml fresh medium was added to make 10 ml and triturated. Cell Count was done before adding cells into the new plates for keeping equal cell numbers in the plates.

Trypan Blue Cell Viability:

After trypsinizing, 10 µL of medium and 10 µL of Trypan Blue dye was prepared in the 1.5ml tube, and 10 uL of this solution was loaded in the slide and slide was inserted, cell viability and cells were counted by Countess II Automated Cell Counter.

Bringing Up cells from Liquid Nitrogen:

The vial of frozen cells was removed from liquid nitrogen and allowed to warm up at room temperature and then directly poured into the plate containing 10mL of fresh medium. Plate was kept in the incubator overnight and on the next day, fresh medium was added to remove cells which were not attached to the plate. Cells were checked every 2-3 days.

Treatments:

For the treatments, 3ml of HBSS+BSA was added into the plate after washing with FPBS twice. The cells were then treated with different fluences of UVB for fluences response curve. For vehicle, 0.1% Ethanol was added in HBSS+BSA. The plates were than kept under UVB blue light for calculated time points (1kJ/m^2- 3 mins and 40 secs, 2.5 kJ/m^2- 9 mins and 17 secs, 5kJ/m^2- 18 mins and 33 secs). Concentration for CPAF was 100 µM stock, 3 µL from stock was added in 3 ml of HBSS+BSA to make the final concentration of 100nM. TPA/PMA was prepared from the vial by first adding 5 ml of ethanol to make the stock, 100 nM was considered as the final concentration and was prepared in HBSS+BSA, 3 ml was added into the plates. For no treatment group, 3 ml of HBSS+BSA was added into the plate. Incubation time was considered four hours.

Microvesicle particles Isolation:

After the designated incubation time, supernatant was transferred from the treatment plates into centrifuge tubes and centrifuged at 2,000 x g for 20 minutes in 4°C. After 20 minutes, supernatant was transferred into new 2 ml centrifuge tubes. It was again centrifuged at 20,000 x g for 70 minutes at 4℃. Supernatant was discarded gently, and tube was placed upside down for 5 minutes so that it dries out completely. The pellet in the tube was resuspended with 100 µL FPBS.

Microvesicle particles Detection:

The NanoSight 300 was used to measure MVP. The samples were diluted with FPBS to remain within the threshold of the NanoSight NS300 instrument. After dilution, 700 µL was loaded by a syringe into the machine and 3 videos for 30 seconds was calculated. The analysis was shown as the mean of 3 tests.

Microvesicle particles Analysis:

The final particle concentration was calculated by considering dilution rate. Fold change was calculated by normalizing treatment group to the no treatment group. In the case of CPAF and TPA, fold change was calculated by normalizing with vehicle. Mean ± S.E. was determined for each group and analyzed on Minitab 19.

MICE:

Experiments was conducted between *XPA* knockout mice (*XPA*-deficient mice), *Smpd1* KO and C57BL/6 wild-type mice. Mice used were aged 10-12 weeks. Mice were received ketamine/xylazine by weight to make them sleep during the treatment. The backs of the mice were shaved, and they received different fluences of UVB by calculated time points (1 kJ/m^2, 2.5 kJ/m^2, 5 kJ/m^2). For CPAF TPA experiment, TPA (100 µM) was topically applied. CPAF was prepared in the saline, the concentration was 250 ng and 250 µL was injected. For vehicle, 90% DMSO:10% ethanol was considered. For UVB + Imipramine experiment, 5 kJ/m^2 UVB fluence was received by shaved mice and 500 µM concentration of imipramine, 100 µL was applied topically. Mice were housed under specific pathogen-free conditions and all mice experiments were according to the protocol of the experimental use of animals approved by Wright State University, School of Medicine's Laboratory Animal Care And Use Committee (LACUC).

The designated time point for all the mice experiment was four hours. Skin biopsy was taken for each mouse by biopsy punch (INTEGRA MILTEX, 5mm). Three biopsies were taken for MVP study, three biopsies for mRNA and stored in RNA Later at 4℃ for overnight, next day it was transferred into -20℃. One biopsy was taken for histology study, they were stored in 10% Formalin at room temperature overnight and on next day, formalin solution was removed and 70% ethanol was added. Blood was collected from each mouse using syringe into heparin blood vacutainer for MVP detection in the blood plasma.

Microvesicle particles Isolation:

- Blood samples were centrifuged at 2000 x g for 20 minutes at 4℃. The supernatant was designated plasma, and the plasma volume was recorded, it was then transferred immediately into a 1.5 ml of centrifuge tube, the tube was filled with 1.5 ml of FPBS and centrifuged at 2000 x g for 20 minutes at 4℃. Supernatant was directly poured into a new 1.5 ml tube. They were spun at 20,000 x g for 10 minutes at 4℃. Supernatant was directly poured into 2 ml of centrifuge tube. They were spin at 20,000 x g for 70 minutes at 4℃. Supernatant was discarded and tubes were placed upside down for at least 5 minutes for air drying. Pellet in the tube was re-suspended with 100 µL of FPBS.
- The skin biopsy was weighed and kept in 1.5 ml of centrifuge tube. In the tubes, 0.5 ml of Collagenase/Dispase (final was added and biopsy was cut finely with surgical scissors. Tubes were placed on shaker at 37℃ overnight.

  Next day, 1.5 ml of FPBS was added in each tube and centrifuged at 2000 x g for 20 minutes at 4℃. Supernatant was directly poured into a new 1.5 ml tube. These tubes were centrifuged at 20,000 x g for 10 minutes at 4℃. Supernatant was directly poured into 2 ml of centrifuge tube. They were spun at 20,000 x g for 70 minutes at 4℃.

Supernatant was discarded and tubes were placed upside down for at least 5 minutes for air drying. Pellet in the tube was re-suspended with 100 µL of FPBS.

Microvesicle particles Detection:

MVPs were detected by NanoSight NS300. The samples were diluted with FPBS to remain within the threshold of the NanoSight NS300 instrument. After dilution, 700 µL was loaded by a syringe into the machine and 3 videos for 30 seconds was calculated. The analysis was shown as the mean of 3 tests.

Erythema Measurements:

Erythema was measured with the help of the Mexameter MX 18. Mexameter was placed on the back of skin and three readings was taken for each mouse before UVB and after the designated incubation period. Differences were taken as a measure of change in erythema.

RNA Isolation and Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR):

RNA was isolated from the skin samples using RNeasy Plus Micro Kit (Cat No. 74034). First strand complementary DNA (cDNA) was generated using 100ng RNA with a Reverse Transcription Kit using random primers (Bio-Rad). Reactions were run in triplicate using Real Time PCR instrument using gene-specific primers. Ct values were determined using the standard 2^-ΔΔCt method.

The following primer sequences were used mouse gapdh 5'-TGCACCACC AACTGCTTAG–3' (forward) and 5'-GATGCAGGGATGATGTTC-3', mouse TNF 5'-TACTGAACTTCGGGGTGATTGGTCC-3' (forward) and 5'-CAGCCTTGTCCCTTGAAGAGAACC-3' (reverse), mouse IL-6 5'-CAGAATTGCCATCGTACAACTCTTTTCTCA-3' (forward) and 5'-AAGTGCATCGTTGTTCATACA-3' (reverse).

STATISTICAL ANALYSIS:

Graphs were made using Microsoft Excel. Mean and significance was determined. Comparison between groups was performed using One - Way Anova on Minitab 19. Data is shown as mean ± S.E of at least three experiments. If the P values were less than 0.05 than the differences between sample were considered as significant. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

CHAPTER IV – RESULTS

UVB generates increased MVP release in HaCaT *XPA* deficient cells.

To determine MVP release in the human keratinocyte derived cell line HaCaT, different fluences of UVB was considered as treatment. Cells were grown and exposed with UVB (1kJ/m^2, 2.5kJ/m^2, 5kJ/m^2). After the determined incubation period, MVP release was analyzed. As shown in Figure- 4 and 5, with increase fluences of UVB, MVP generation was also recorded higher with decreased percentage cell viability. Also, MVP release was significantly higher in HaCaT *XPA* deficient cells compared to HaCaT cells.

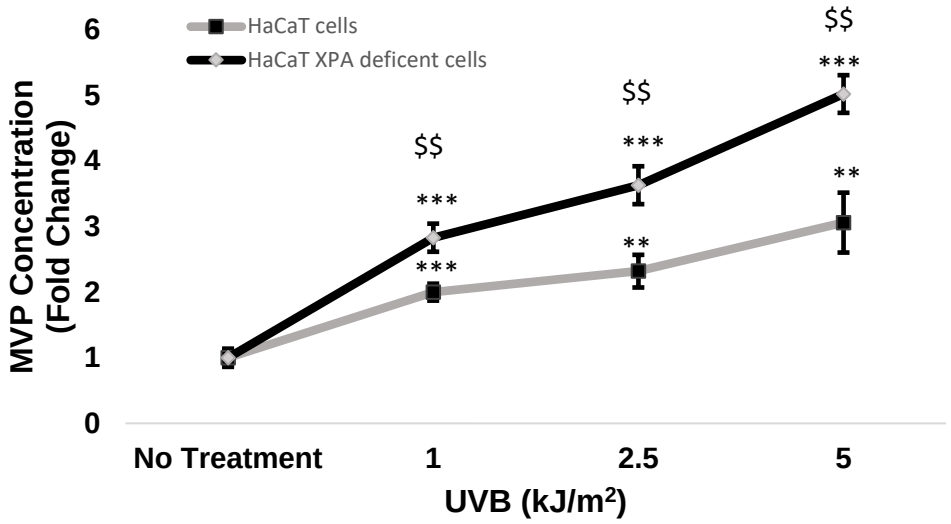


Figure 4: UVB generates increased MVP release in HaCaT *XPA* deficient cells. HaCaT cells and HaCaT *XPA* deficient cells received no treatment (NT), 1kJ/m^2, UVB 2.5kJ/m^2 and 5kJ/m^2. After 4 hours, supernatant was removed and MVP was measured. The numbers of MVP were normalized to cell count and NT. Data are the mean ± S.E. fold change of at least three experiments. Groups were compared using One-Way Anova. ($P < 0.01$) ($P < 0.001$) was considered statistically significant. ** and *** denotes changes between NT and UVB. $$ denotes changes between HaCaT and HaCaT *XPA* deficient cells.

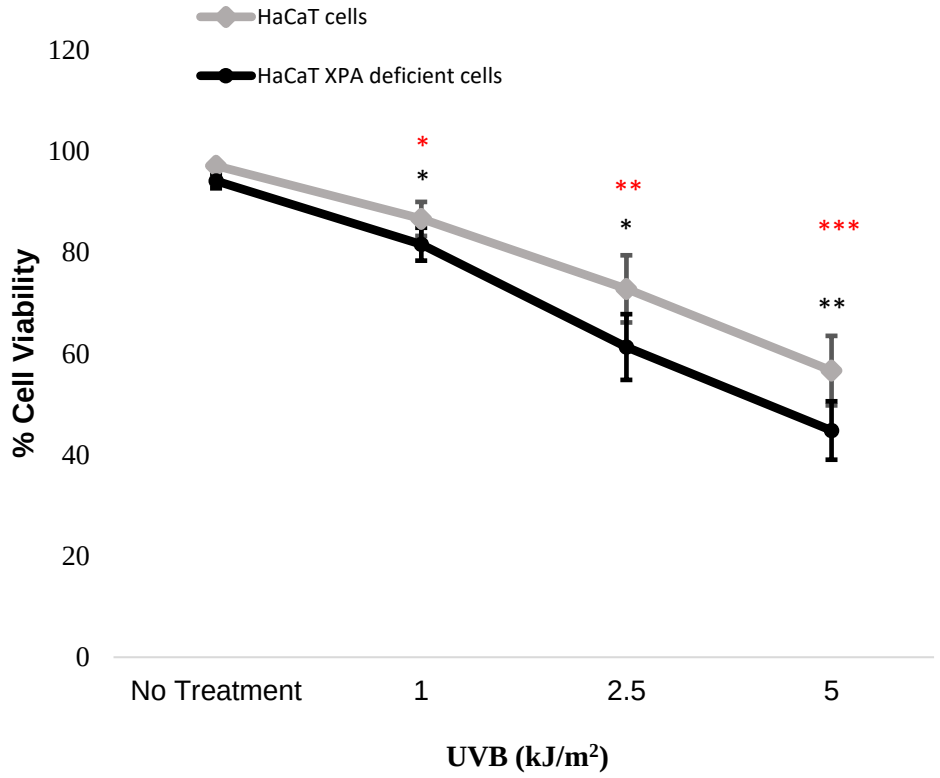
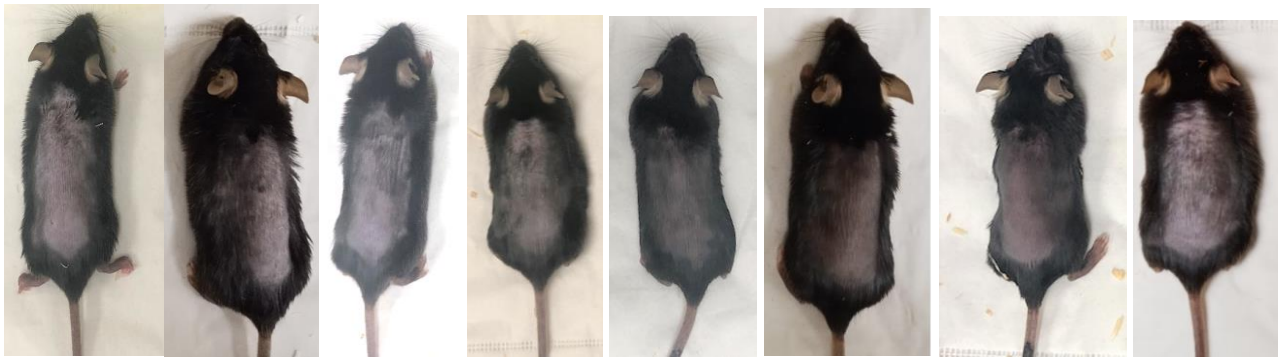


Figure 5: UVB irradiation decreases percentage cell viability in HaCaT and HaCaT *XPA* deficient cells. After four hours of UVB treatment, cells were trypsinized and cell viability was determined by Countess II Automated Cell Counter. (A) HaCaT cells and (B) HaCaT *XPA* deficient cells, data are the mean ± S.E. of at least three experiments. Groups were compared using One-Way Anova and Tukey's post-hoc test. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) changes between NT and UVB treatment group; no statistically significant difference between HaCaT *XPA* deficient and HaCaT cells in decrease cell survival rate for UVB treatment**.** Red indicates for *XPA* deficient cells.

UVB irradiation generates greater erythema and MVPs release in *XPA* KO mice

To confirm that increase fluences of UVB treatment might release significantly higher MVP in *XPA* KO mice than WT mice, mice were anesthetized and their shaved back were irradiated with UVB (1kJ/m^2, 2.5kJ/m^2, 5kJ/m^2). After 4 hours of designated period, skin biopsies were taken and blood plasma was collected from each mouse for MVP analysis. To see if UVB irradiation can cause redness on the back of mice, pictures were taken and erythema was measured with the help of Mexameter before UVB treatment and after 4 hours of UVB treatment. As shown in Figure-6, erythema measurements in *XPA* KO mice were higher than WT mice after the UVB irradiation. MVP generation was increased significantly in skin tissue and blood plasma (Figure-7). In skin tissue and blood plasma, MVP generation was twice as high in *XPA* KO mice than WT mice with increasing UVB fluences. As previously shown in *XPA* deficient murine model, UVB treatment increases formation of Ox-GPC and PAF-R (Yao, 2012), because of higher levels of PAF-R, MVP generation was highly increased in photosensitive model.

(A).



WT NT *XPA* KO NT WT UVB *XPA* KO UVB WT UVB *XPA* KO UVB WT UVB *XPA* KO UVB

1kJ/m^2 2.5kJ/m^2 5kJ/m^2

(B).

(C).

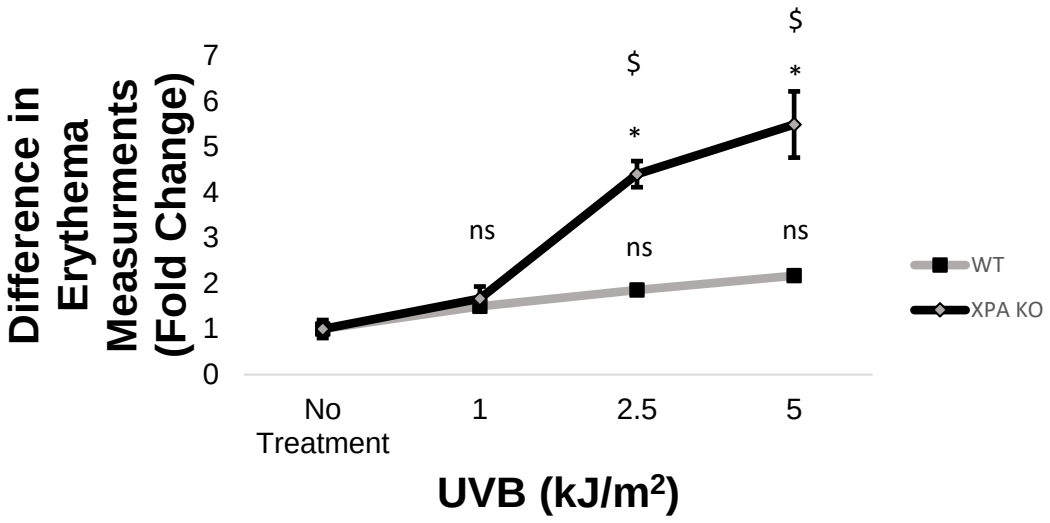
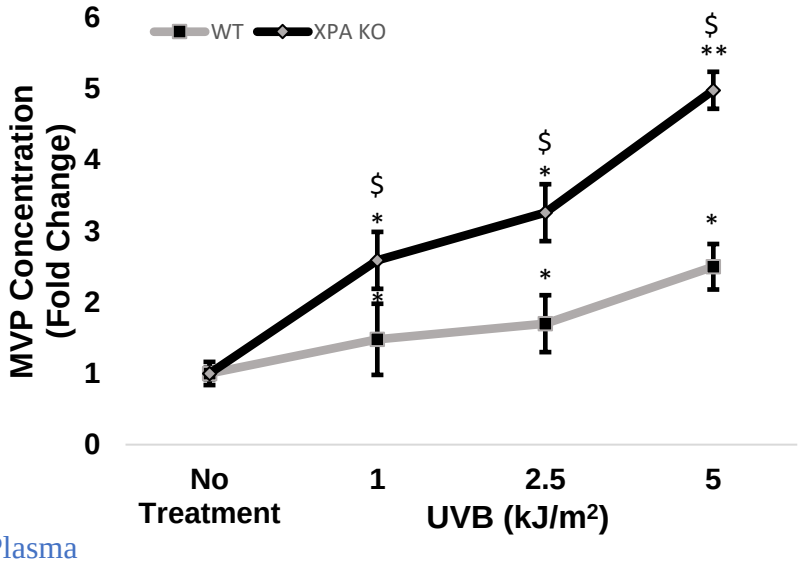


Figure 6: UVB irradiation generates greater erythema response in *XPA* KO mice. Groups of 8-13 wild-type (C57BL/6) and *XPA* KO mice were either received NT, 1 kJ/m^2 of UVB, 2.5 kJ/m^2 of UVB and 5 kJ/m^2 of UVB to dorsal back skin and were analyzed four hours post-treatment. (A) Representative photographs depicting erythema in Wildtype and *XPA* KO mice. (B) Picture of Mexameter MX 18 showing an example for Erythema readings. (C) Before and four hours of post-UVB treatment, erythema was measured with Mexameter. Data is shown as mean ± S.D. of fold change in erythema readings. Groups were compared using One-Way Anova. $P < 0.05$ was considered statistically significant. ($) denotes changes between wild-type and *XPA* KO mice. (* and non-significant (ns)) denotes changes between no treatment and UVB.

(A). Skin Tissue



(B). Plasma

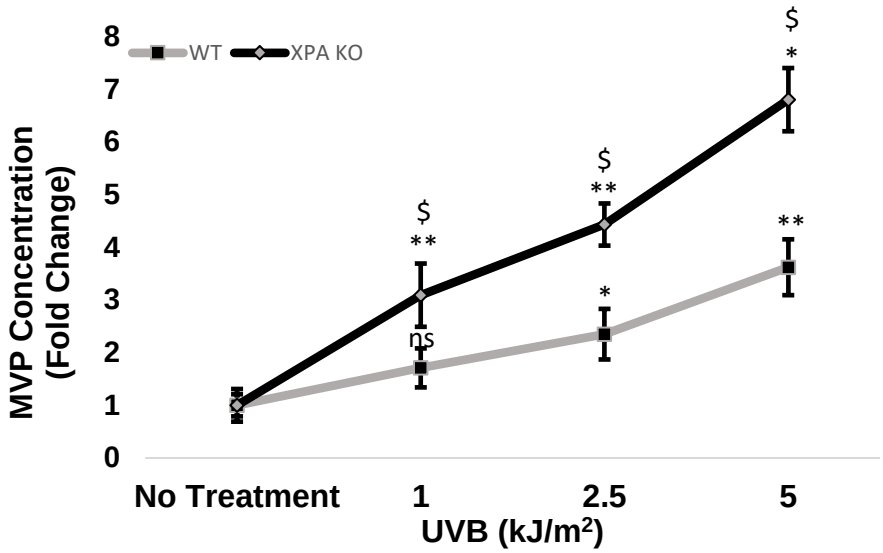
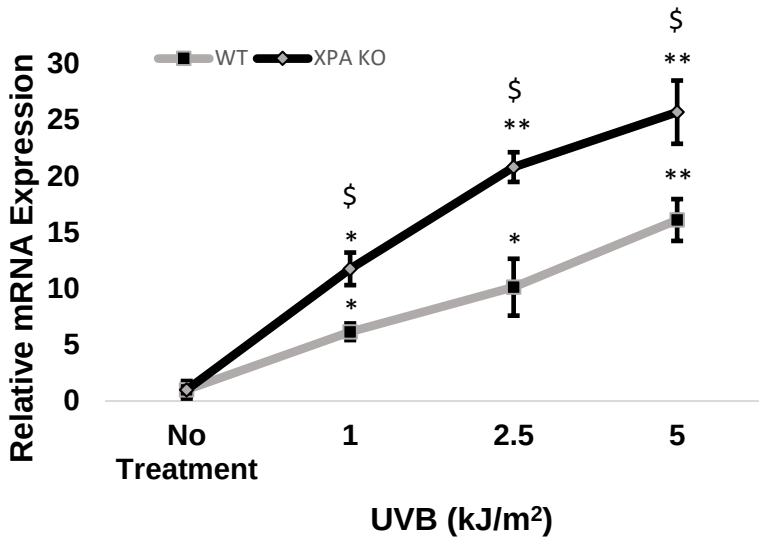


Figure 7: UVB irradiation generates increased MVP release in *XPA* KO mice. Groups of 8-13 wild-type (C57BL/6) and *XPA* KO mice were either treated with NT, 1 kJ/m^2 of UVB, 2.5 kJ/m^2 of UVB and 5 kJ/m^2 of UVB to dorsal back skin. Four hours post-treatment, (A). Triplicate skin biopsies were obtained, weighed and MVP was measured. (B). Plasma was obtained and MVP was measured. Data are the mean ± S.E. fold change of MVP count. Groups were compared using One-Way Anova and Tukey's post-hoc test. Differences in samples were considered significant if the P value is less than 0.05. P < 0.05 ($) changes between WT and *XPA* KO mice; P < 0.05 (*) P < 0.01 (**) changes between NT and UVB treatment.

(A). TNF-alpha



(B). IL-6

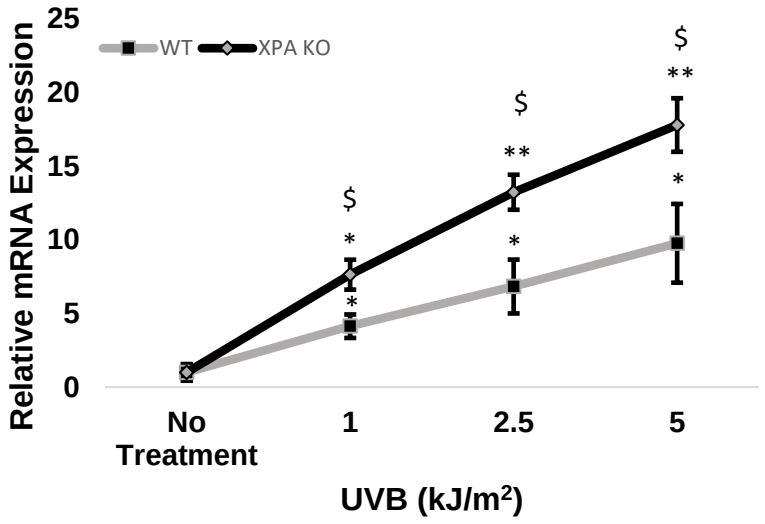


Figure 8: UVB irradiation generates increase pro-inflammatory cytokines in *XPA* KO mice. RNA was isolated from skin biopsies of wild-type and *XPA* KO mice and RT-qPCR was performed. Data are the mean ± S.E. relative mRNA expression of (A) TNF-alpha and (B) IL-6. Groups were compared using One-Way Anova and Tukey's post-hoc test. Differences in samples were considered significant if the P value is less than 0.05. $P < 0.05$ ($) changes between WT and *XPA KO* mice; $P < 0.05$ (*) $P < 0.01$ (**) changes between NT and UVB.

UVB irradiation generates increase pro-inflammatory cytokines in *XPA* KO mice.

RNA was isolated from skin biopsy and RT-qPCR was performed for TNF and IL-6. Environmental stressor UVB, causes upregulation of TNF and IL-6 in WT and *XPA* KO mice (Figure-8). However, inflammatory cytokines were more highly expressed in photosensitive mice (*XPA* KO) as compared to WT mice. These data suggest that MVP released in extracellular environment might be responsible in upregulation of cytokines. Hence, more MVP release result in more cytokine disbalance in the environment. Thus, MVP might act as a regulator for causing inflammation in photosensitive conditions.

CPAF/TPA treatment does not result in differences in MVP release in HaCaT *XPA* deficient cells vs HaCaT cells.

To see whether ROS production is involved in the increased generation of MVP, HaCaT cells and HaCaT *XPA* deficient cells were grown and treated with UVB (1kJ/m^2, 5kJ/m^2), CPAF 100nM (Carbamyl-platelet-activating factor (CPAF) an analogue of PAF. CPAF acts as PAF agonist by increasing free intracellular calcium levels (Travers JB L. Q., 1992), TPA/PMA 100nM (tetradecanoyl phorbol acetate (TPA) known for stimulation of MAPK), Vehicle (HBSS+BSA:EtOH) and control was considered. After 4 hours of incubation period, MVP was isolated and analyzed. As shown in Figure-9, HaCaT cells or HaCaT *XPA* deficient cells, treated with CPAF or TPA (agents which do not induce ROS production) were showing no difference in MVP release, while UVB (agent that induce ROS) treated HaCaT *XPA* deficient cells were able to generate MVP higher than HaCaT cells. Also, UVB treated cells have shown reduced cell survival percentage as compared to CPAF TPA treated cells (Figure-10).

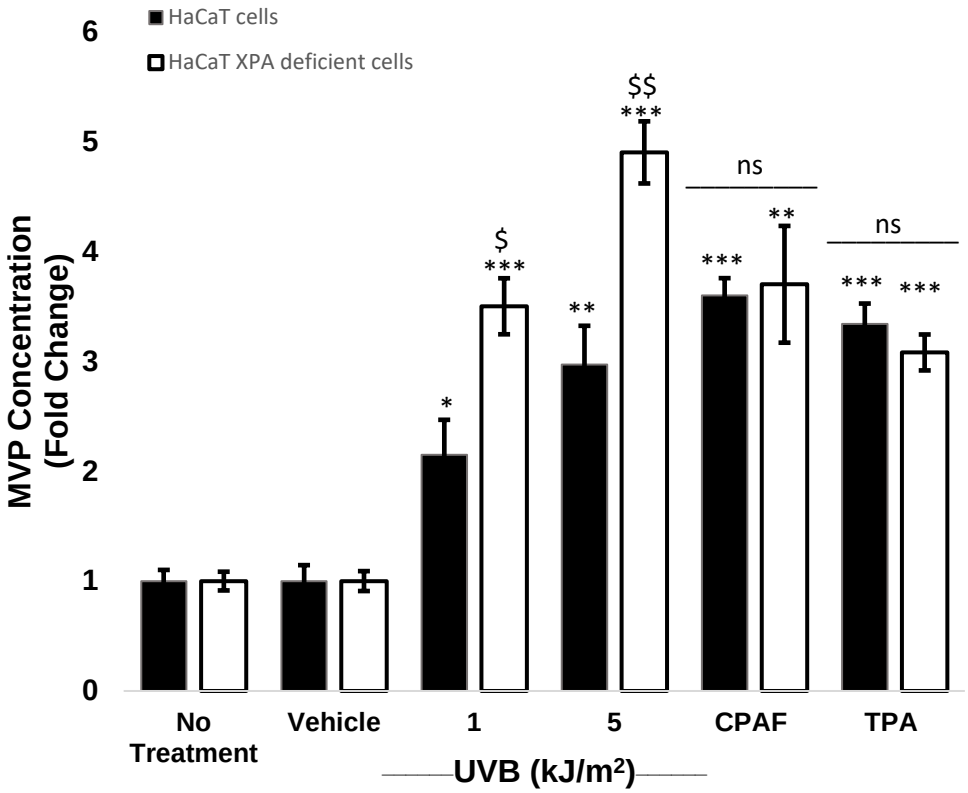


Figure 9: CPAF/TPA treatment does not result in differences in MVP release in HaCaT *XPA* deficient cells vs. HaCaT cells. HaCaT cells and HaCaT *XPA* deficient cells were either received No Treatment, Vehicle (0.1% EtOH), 1kJ/m^2 UVB, 2.5kJ/m^2 UVB, CPAF 100nM and TPA 100nM. Supernatant was removed four hours post-treatment and MVP was quantitated and normalized to cell counts and no treatment. Data are the mean ± S.E. fold change of MVP from at least three separate experiments. Groups were compared using One-Way Anova and Tukey's post-hoc test. Differences in samples were considered significant if the P value is less than 0.05. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and non-significant (ns) changes between No Treatment and UVB; Vehicle and CPAF , TPA treatment. $P < 0.05$ ($), $P < 0.01$ ($$) and non-significant (ns) changes between HaCaT and HaCaT XPA deficient cells.

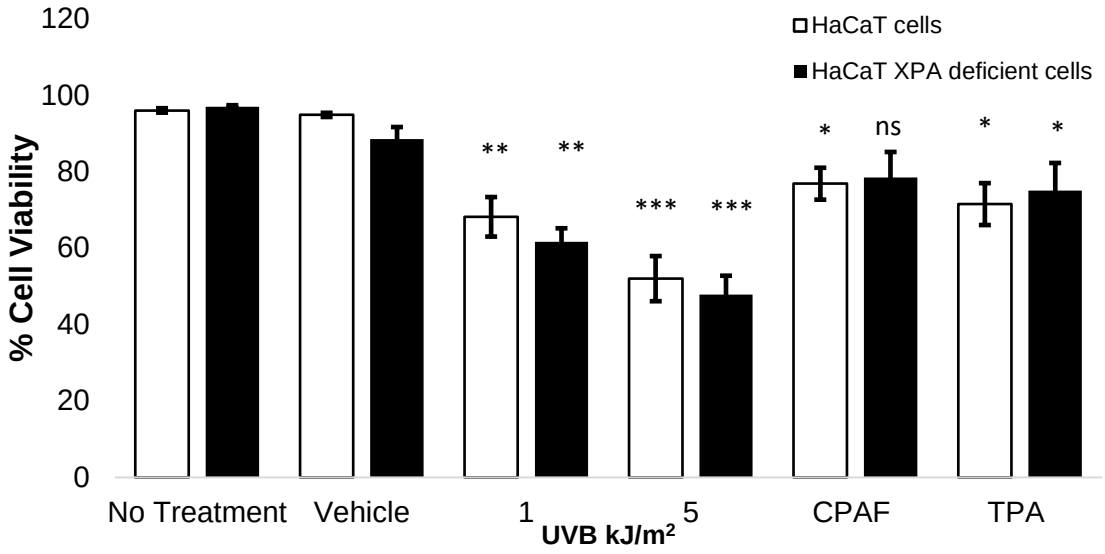
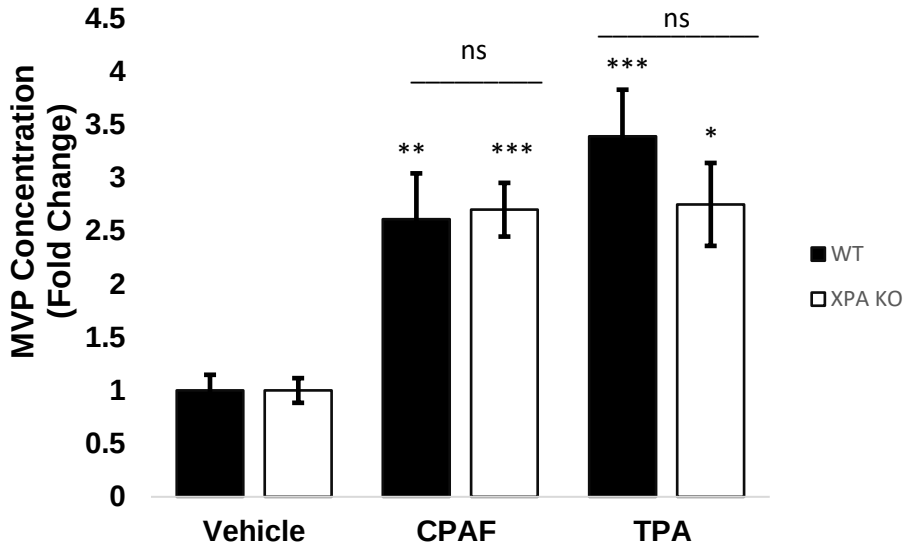


Figure 10: Effect of various treatments on percentage cell viability of HaCaT and HaCaT XPA deficient cell lines. After the treatments shown in figure 9, cells were trypsinized and cell viability was determined with Countess II. (A) HaCaT cells and (B) HaCaT XPA deficient cells, data are the mean ± S.E. of at least three experiments. Groups were compared using One-Way Anova and Tukey's post-hoc test. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and nonsignificant (ns) changes between No Treatment and UVB; Vehicle and CPAF 100 nM or TPA 100 nM in HaCaT and HaCaT XPA deficient cells.

(A). Skin Tissue



(B). Plasma

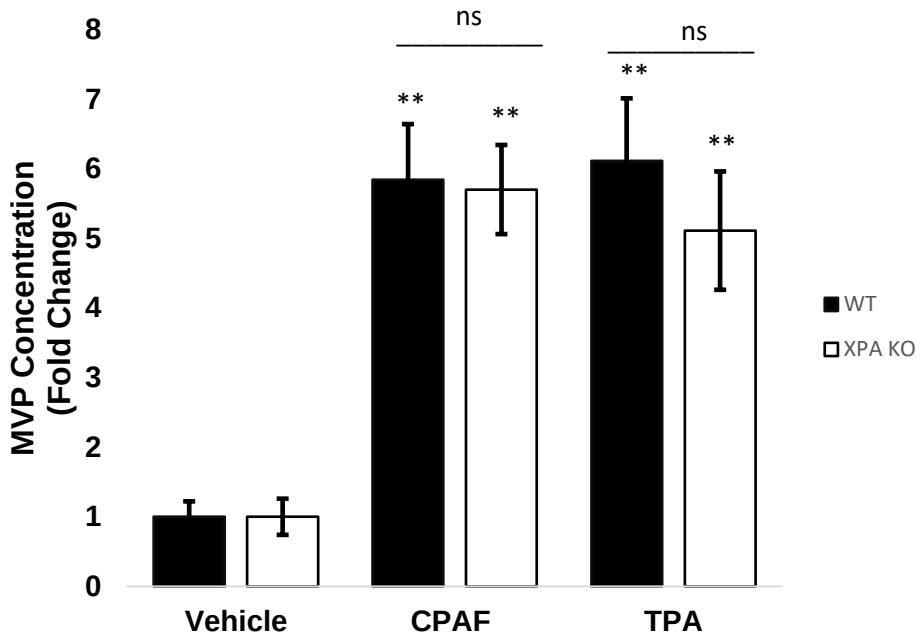


Figure 11: CPAF/TPA treatment does not result in differences in MVP release in Wild-type vs. *XPA* KO mice. Groups of 7-9 Wild-type and *XPA* KO mice have their backs shaved and treated with 90% DMSO (Vehicle), TPA 100 µM topically and injected CPAF 250ng i.p. Four hours post-treatment, (A). Triplicate skin biopsies were obtained and MVP was measured.(B). Plasma was obtained and MVP was measured. Data are the mean ± S.E. fold change of MVP. Groups were compared using One-Way Anova and Tukey's post-hoc test. Differences in samples were considered significant if the P value is less than 0.05. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) between Vehicle and CPAF/TPA and non-significant (ns) changes between WT and *XPA* KO mice.

CPAF/TPA treatment does not result in differences in MVP release in *XPA* KO mice vs WT mice.

To confirm if there is a difference in MVP generation between WT and *XPA* KO mice when treated with PAF agonist (CPAF) or TPA that does not generate MVP by stimulating ROS, shaved back of anesthetized mice were injected with 250 microL of CPAF (250 ng) i.p. and 100 microL of TPA (100 microM) was applied topically on shaved back of mice and for vehicle (90% DMSO) was also applied topically. After 4 hours, skin biopsy was taken and blood plasma was collected and MVP was analyzed. In skin tissue or blood plasma, there was no difference in MVP release between WT and *XPA* KO mice (Figure-11). These data confirm that, model that lacks *XPA* and the one that does not lack *XPA*, produce UVB – MVP by inducing ROS and CPAF or TPA directly activates PAF-R or stimulate other pathways to generate MVP without inducing ROS. Hence, ROS production is involved in generation of MVP release by UVB and irregular ROS production can cause increase microvesicle release in photosensitive condition.

Acid sphingomyelinase Inhibitor imipramine reduces UVB induced erythema and MVP release in *XPA* KO mice.

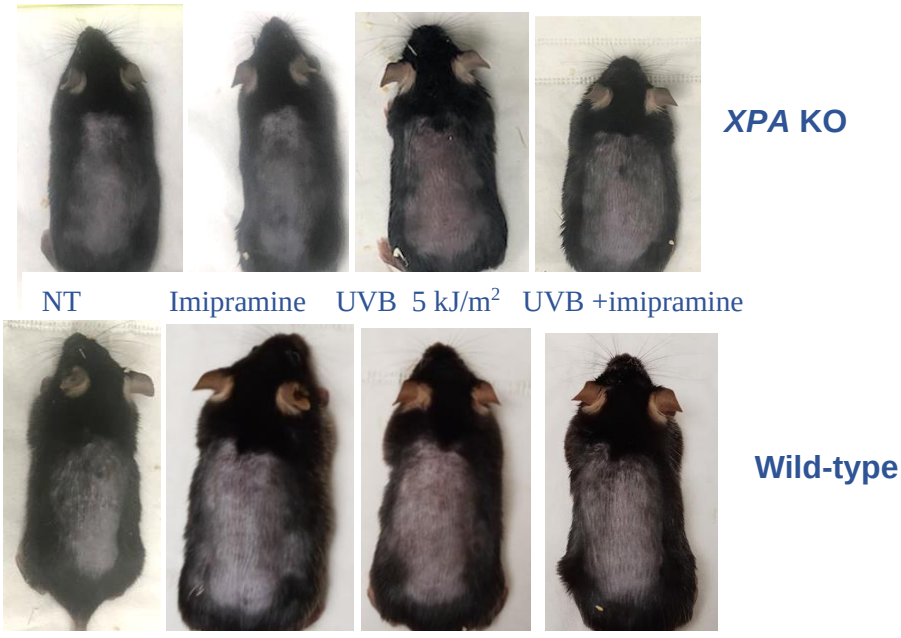
Acid Sphingomyelinase (aSMase) is also known to generate MVP via activation of PAF-R. Inhibitors of aSMase might be a novel strategy to block UVB-MVP release in *XPA* deficient model. Imipramine (a known aSMase inhibitor) has shown to have an inhibitory effect on MVP generation. To see whether Imipramine can block UVB-MVP release in *XPA* deficient murine model, anesthetized WT and *XPA* deficient mice were irradiated with 5 kJ/m^2 UVB and immediately after UVB treatment, 100 microL of imipramine (500 microM) was applied topically on the shaved back of

mice. To check the inhibition of MVP release by Imipramine, mice were also treated with only UVB fluences and to make sure if Imipramine alone is not causing MVP generation, imipramine alone mice group was also considered and lastly, control group was considered that does not received UVB or imipramine. Erythema was measured by Mexameter before treatment and after 4 hours of treatment. Skin biopsy and blood plasma was collected and MVP was analyzed. As shown in Figure-12, UVB treatment alone showed greater erythema which was reduced significantly after applying imipramine topically. aSMase inhibitor imipramine was able to block MVP release after the UVB treatment in photosensitive mice and Wild Type mice (Figure-13). However, UVB treatment alone group was showing 5-to-8-fold increase in MVP generation in *XPA* KO mice than WT mice (Figure-13). Imipramine alone has minimal effect on generation of MVP.

Acid Sphingomyelinase Inhibitor reduces UVB induced pro-inflammatory cytokines in XPA KO mice.

To confirm if imipramine can reduce pro-inflammatory cytokine production induced by UVB, RNA was isolated from skin biopsy and RT-qPCR was performed. TNF and IL-6 were upregulated by UVB were significantly downregulated by topically applied Imipramine (Figure- 14). These data show that, pharmacologic approach to block MVP release might be a novel strategy in the treatment of photosensitivity.

(A).



(B).

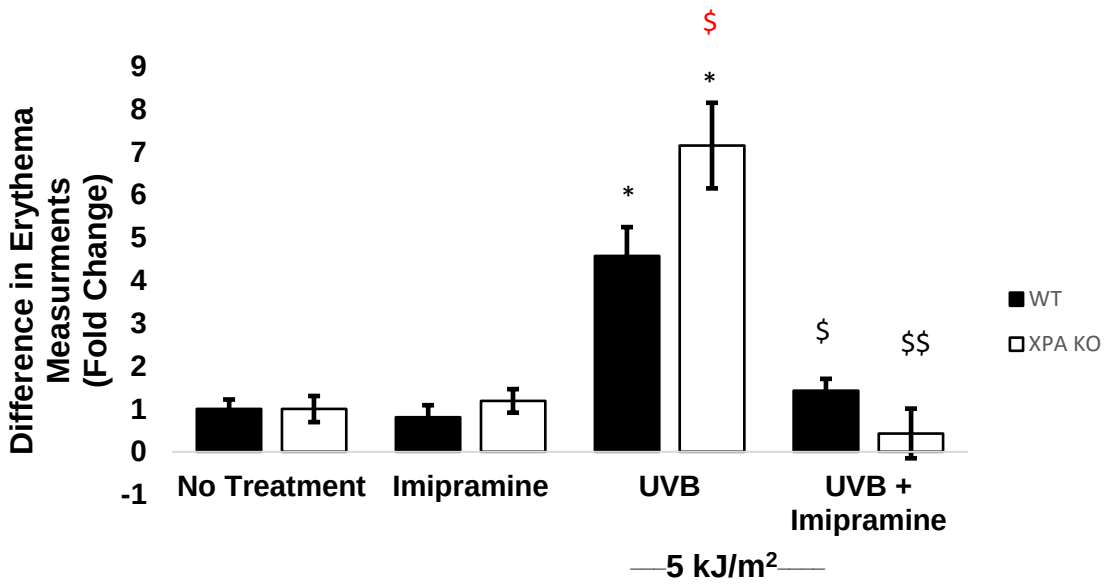
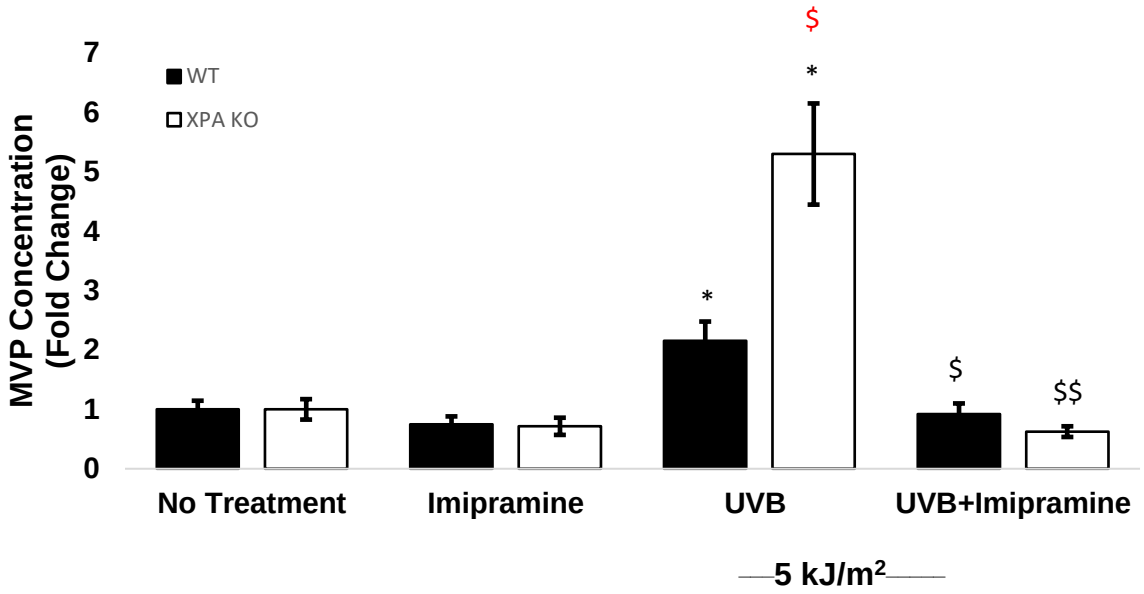


Figure 12: Acid Sphingomyelinase inhibitor imipramine reduces UVB induced erythema in *XPA* KO mice. Group of 6-8 wild-type and *XPA* KO mice were either received no treatment (NT), imipramine 500 µM only, 5kJ/m^2 UVB, UVB + imipramine 500 µM. (A) Representative photographs showing comparison of redness on shaved back of WT and *XPA* KO mice between UVB treatment group and UVB + imipramine treatment group. (B) Before and after four hours of post-treatment erythema was measured with Mexameter MX 18. Data is shown as mean ± S.E. of fold change in erythema measurements. P < 0.05 (*) between NT and UVB. P < 0.01 ($$) changes between UVB only and UVB + imipramine in both Wildtype and *XPA* KO mice and P < 0.05 ($) between UVB group in WT and *XPA* KO mice.

(A). Skin Tissue



(B). Plasma

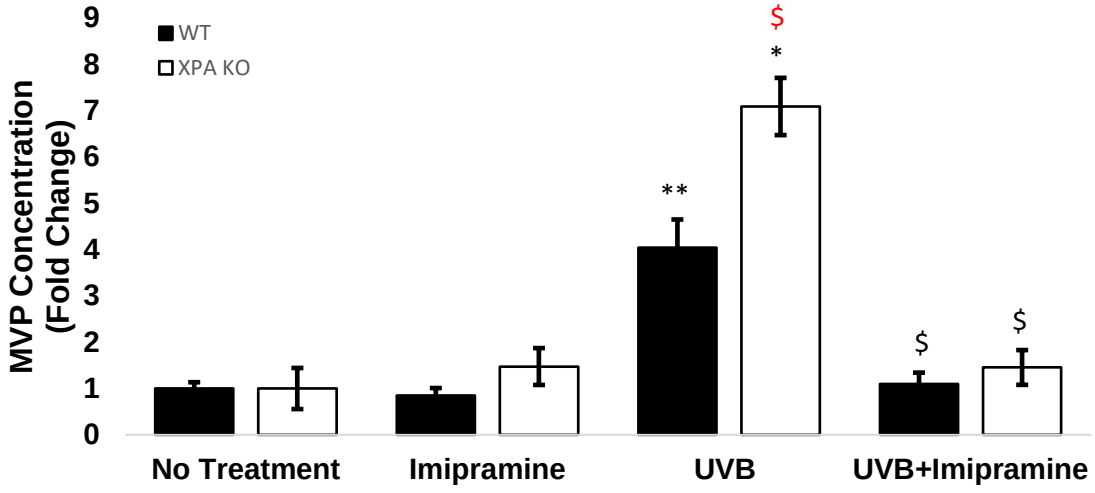
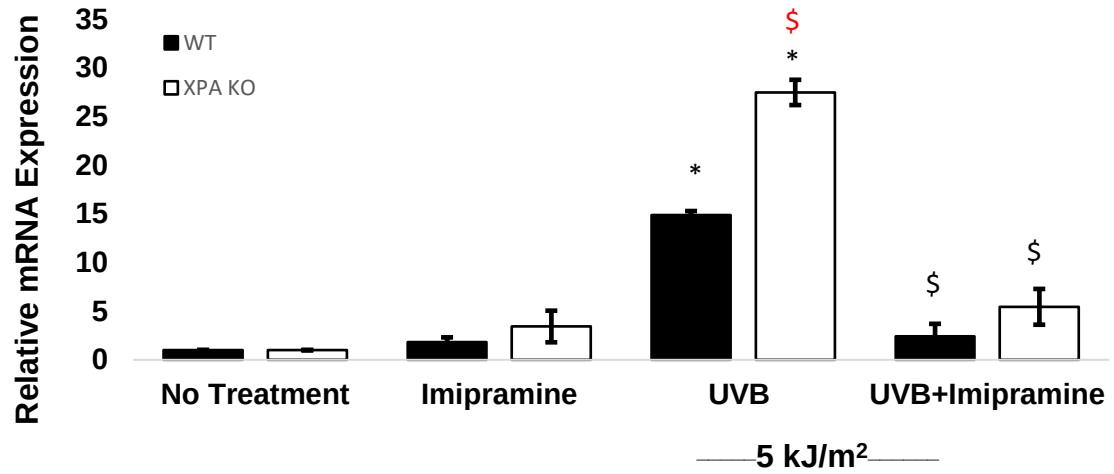


Figure 13: Acid Sphingomyelinase inhibitor imipramine inhibits UVB induced MVP release in *XPA* KO mice. Groups of 6-8 wild-type and *XPA* KO mice were either received no treatment (NT), imipramine 500 µM only, 5KJ/m2 UVB, UVB + imipramine 500 µM and were analyzed after 4 hours of post-treatment. (A) Triplicate skin biopsies were obtained and MVP was measured. (B) Plasma was obtained and MVP was measured. Data is shown as mean ± S.E. of fold change in MVP count. $P < 0.05$ (*), $P < 0.01$ (**) between NT and UVB. $P < 0.05$ ($), $P < 0.01$ ($$) changes between UVB only and UVB + imipramine in both WT and *XPA* KO mice and $P < 0.05$ ($) between UVB group in WT and *XPA* KO mice.

(A). TNF-alpha



(B). IL-6

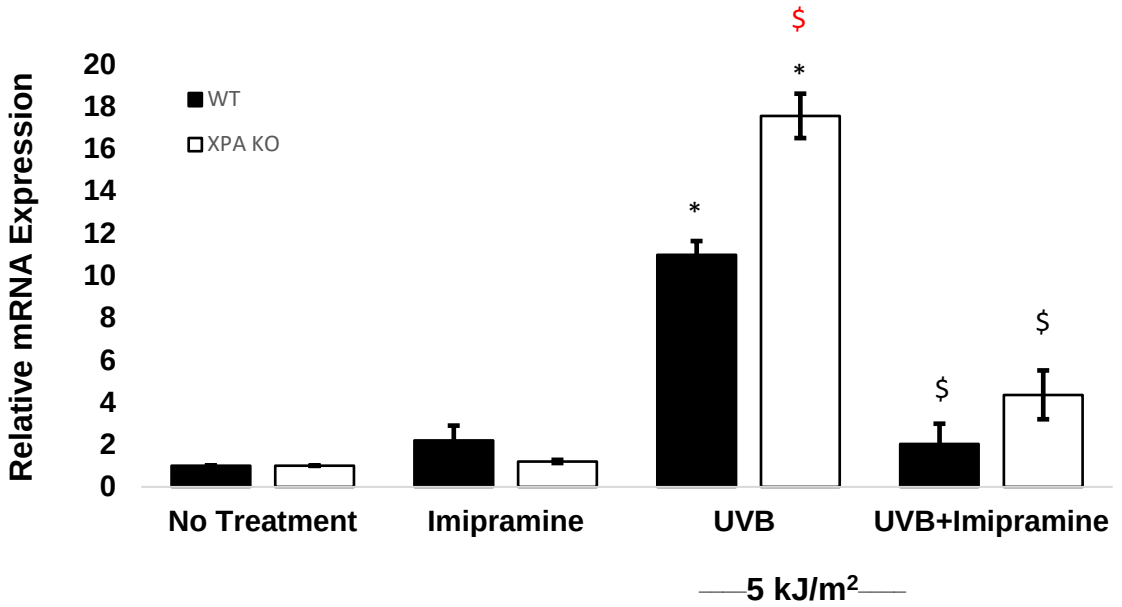


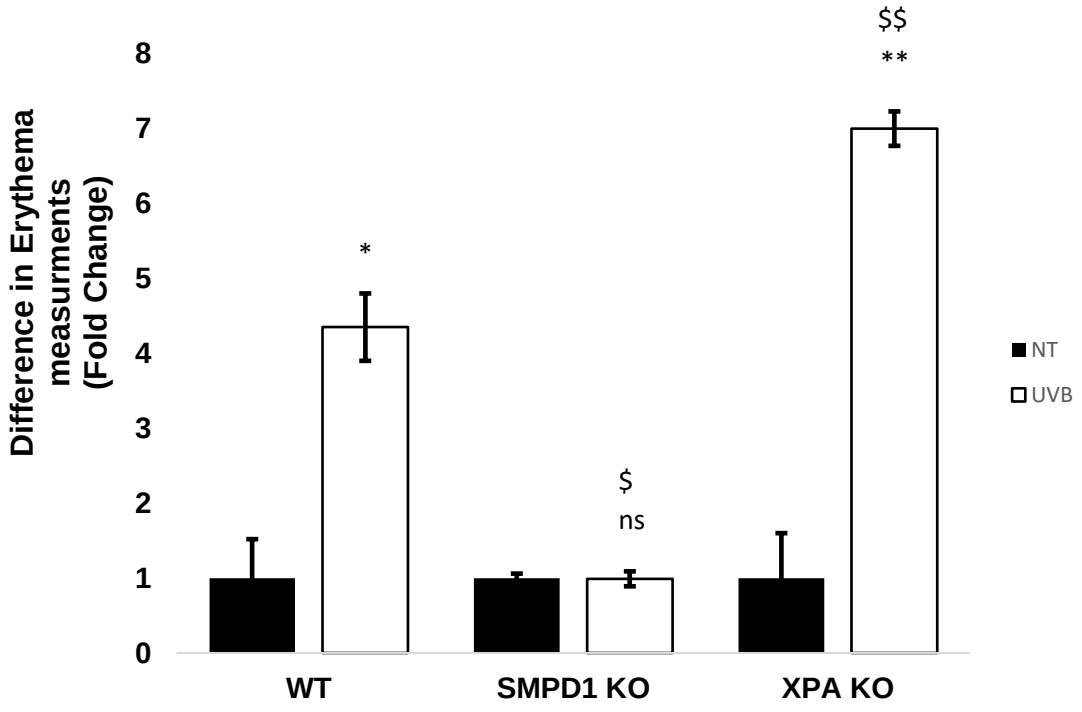
Figure 14: Acid Sphingomyelinase inhibitor imipramine inhibits UVB induced pro-inflammatory cytokines in *XPA* KO mice. RNA was isolated from skin biopsies of Wildtype and *XPA* KO mice and RT-qPCR was performed. Data are the mean ± S.E. relative mRNA expression of (A) TNF-alpha and (B) IL-6. Groups were compared using One-Way Anova. Differences in samples were considered significant if the P value was less than 0.05. $P < 0.05$ (*) between NT and UVB. $P < 0.05$ ($) changes between UVB only and UVB + imipramine in both WT and *XPA* KO mice and $P < 0.05$ ($) between UVB group in WT and *XPA* KO mice.

Role of Acid Sphingomyelinase in UVB induced inflammatory response.

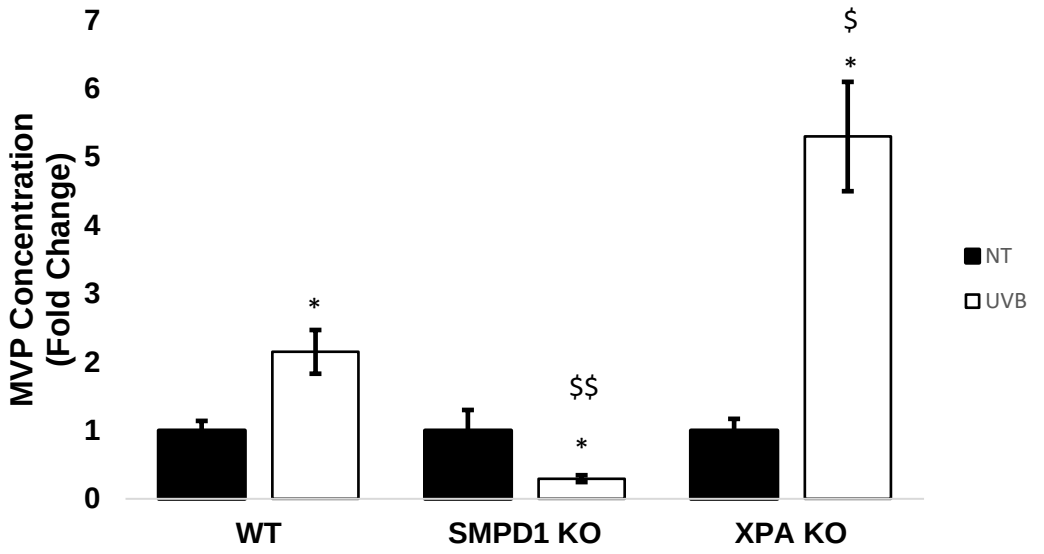
To see whether genetically knocking down the aSMase in mice can be able to inhibit UVB induced MVP release, shaved back of *Smpd1* KO mice along with WT and *XPA* KO mice was irradiated with 5 kJ/m^2 of UVB. After four hours of UVB treatment, erythema was measured and MVP was isolated and detected in skin tissue and plasma. Erythema readings were not much affected with UVB irradiation in comparison to no treatment group in *Smpd1* KO mice, however, erythema was higher in WT and *XPA* KO mice with UVB treatment compared to no treatment (Figure-15(A)). UVB-MVP release was reduced in *Smpd1* KO mice than the no treatment group both in skin tissue and blood plasma (Figure-15(B and C)).

Together, these data indicate that aSMase is required for UVB induced MVP release in either WT or *XPA* KO mice and by inhibiting aSMase by its inhibitor imipramine or genetically knocking down aSMase can decrease the generation of MVP in *XPA* KO mice.

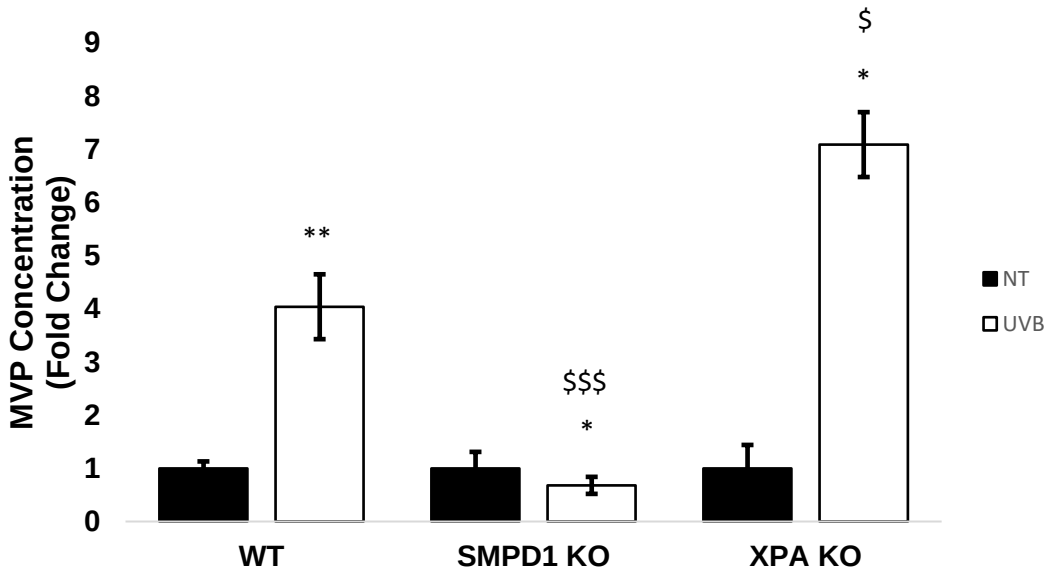
(A). Erythema



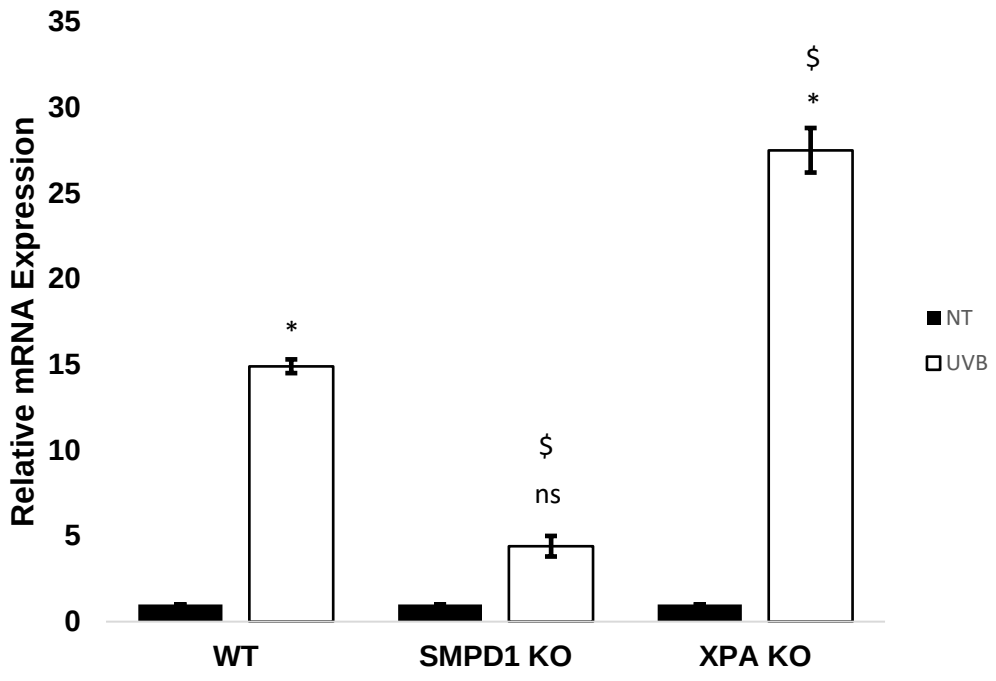
(B). Skin Tissue



(C). Plasma



(D). TNF-alpha



(E). IL-6

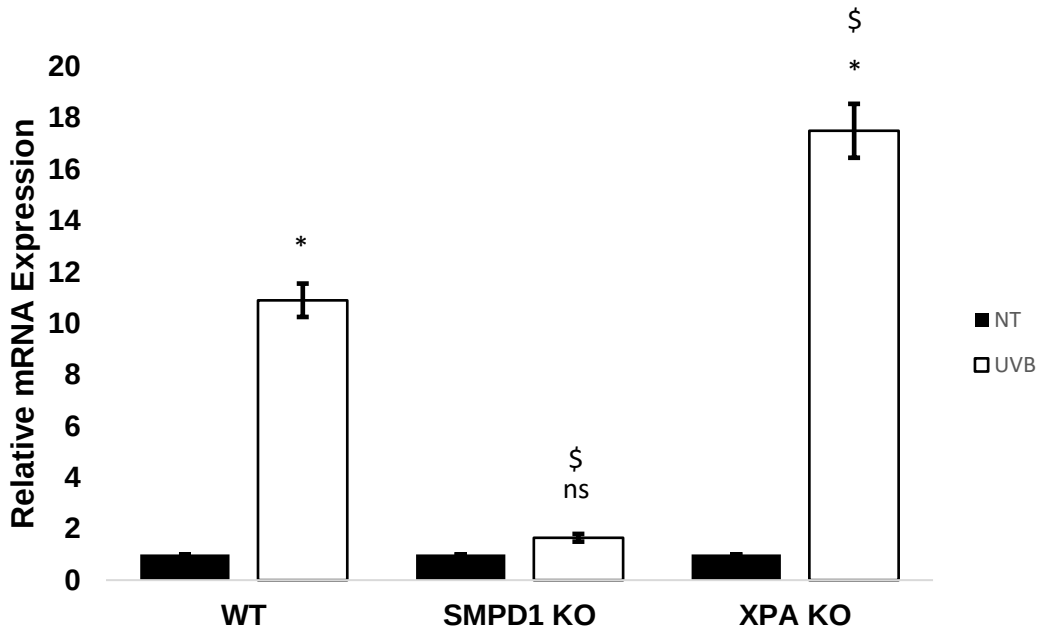


Figure 15: Role of Acid Sphingomyelinase in UVB induced erythema, MVP release and cytokines production. Groups of 7-9 wild-type (WT), *Smpd1* KO and *XPA* KO mice received either no treatment (NT) or 5 kJ/m^2 of UVB. (A) Before and after four hours of post treatment erythema was measured by Mexameter MX 18. Data is shown as mean ± S.D. of fold change in erythema measurements. (B) Triplicate skin biopsies were obtained and MVP was measured. (C) Plasma was obtained and MVP was measured. Data is shown as mean ± S.E. of fold change in MVP. RNA was isolated from skin biopsies of Wildtype, *Smpd1* KO and *XPA* KO mice and RT-qPCR was performed. Data are the mean ± S.E. relative mRNA expression of (D) TNF-alpha and (E) IL-6. $P < 0.05$ (*), $P < 0.01$ (**), non-significant (ns) changes between NT and UVB in WT, *Smpd1* KO and *XPA* KO mice. $P < 0.05$ ($), $P < 0.01$ ($$), $P < 0.001$ ($$$) between WT UVB group and *Smpd1* KO UVB; *XPA* KO UVB treatment group.

CHAPTER V – DISCUSSION

Xeroderma Pigmentosum A protein forms a complex with other proteins involved in Nucleotide Excision Repair and this protein complex removes the damage section of DNA (Yao, 2012). *XPA* deficiency can cause severe sensitivity to Ultraviolet Radiation and increase risk of multiple skin cancer (Kunisada, 2017). However, the exact mechanism through which exaggerated redness and inflammation occurs in *XPA* deficiency by UV radiation is not well elucidated. Previously, it has been shown that PAF a well-known mediator for UV induced inflammation and immunosuppression and Ox-GPCs and PAF-R agonists are elevated in and thus could act as a mediator in photosensitivity associated with *XPA* deficiency (Yao, 2012) (Damiani E, 2016). The present studies involve exaggerated release of microvesicle particles in response to Ultraviolet B Radiation associated with *XPA* deficiency. This study investigated that release of microvesicle particles was increased and pro-inflammatory cytokines were also highly upregulated after UVB irradiation in *XPA* KO in comparison WT group both *in vitro* and *in vivo* which were inhibited by acid sphingomyelinase inhibitor imipramine. To better study effects such as inflammation followed by cell death of UVB irradiation on human skin, we used human immortalized keratinocytes cell line (HaCaT) where shRNA was used for silencing *XPA* gene (Kemp, 2016). From fluences response studies (Figure-4), 5 kJ/m^2 UVB light was seen to produce extreme effects on HaCaT cell line and with increasing UVB irradiation, microvesicle generation was increased significantly in HaCaT *XPA* deficient cells compared to control HaCaT cells (Figure-4). These toxic effects might be because of UVB induces cytokines/chemokines (IL-6, IL-8, TNF-alpha, Interferon gamma) production in human keratinocytes (Yoshizumi M, 2008). UVB irradiation generates inflammatory responses in *XPA* knockout mice by activating certain pathways and genes such as chemokine signaling pathway (CXCL14, CXCL1, CXCL2), cytokine and inflammatory response pathway (CXCL1, IL-6 and tumor necrosis factor) (Yao, 2012) (Kunisada, 2017). Our study confirms that UVB irradiation on the shaved back of wild-type and *XPA* knockout mice generates inflammatory response (Figure-6,). However, *XPA* knockout mice have shown to

produce increased inflammatory response by generating increase microvesicle and cytokines release (Figure-7,8). This response was due to increase production of reactive oxygen species and PAF agonists. To demonstrate if UVB is involved in the increased generation of MVP in *XPA* KO group by inducing higher production of ROS, CPAF/TPA treatment was considered as CPAF/TPA produces MVP by not inducing ROS as they directly act on activating PAF-R or by triggering different pathways in the process to generate MVP release. Our data was able to show that there was no significant difference between WT and *XPA* KO group in MVP release with CPAF/TPA treatment (Figure- 9,11).

Ceramide is known to be a key regulator for releasing MVP through lipid pathway and platelet activating factor is considered to be contributing to generating Acid Sphingomyelinase dependent ceramide (Goñi FM, 2014). Here, we show that either using aSMase inhibitor or by genetically knocking down *Smpd1* gene (acid sphingomyelinase is the protein encoded by *Smpd1* gene) affects the UVB induced production of MVP release and production of pro-inflammatory cytokines (Figure-12-15).

In summary, the present studies indicate that UVB induces increased generation of MVP in *XPA* deficient model both *in vitro* as well as *in vivo* by trigging production of ROS and PAF-R agonists that can be inhibited by aSMase inhibitor or genetically knocking down *Smpd1* gene.

CHAPTER VI – FUTURE STUDIES

The significance of these studies is to demonstrate role of MVP release in photosensitive condition and providing pharmacologic and genetic approach to inhibit MVP release. If future studies include crossing *XPA* KO mice with *Smpd1* KO (*XPA* -/- x *Smpd1* -/-) that will confirm the role of aSMase in UVB induced generation of MVP *in vivo*. These studies, involving MVP and aSMase might be considered as an innovative findings for Lupus Erythematosus where photosensitivity has considered to be major symptom in developing lupus.

CHAPTER VII – CONCLUSION

These studies demonstrate that UVB irradiation induces exaggerated inflammatory responses in *XPA* KO model. These “photosensitive” responses include enhanced erythema, cytokine expression which might be because of increase ROS production and PAF-R agonists that releases MVP by stimulating aSMase. However, these inflammatory responses can be inhibited by topical application of aSMase inhibitor imipramine pharmacologically or by genetically knocking down aSMase. These studies could be considered as groundbreaking research for photosensitive conditions.

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