Comparison between 8-Methoxypsoralen and 5-Aminolevulinic Acid in Killing T Cells of Photopheresis Patients *ex vivo*

Running title: 8-MOP versus 5-ALA for *ex vivo* photopheresis

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Conflict of Interest Statement (COI)

The authors declare no conflicts of interest with the contents of this study.

Abstract

Background and Objective: Extracorporeal photopheresis (ECP), an established modality for cutaneous T-cell lymphoma (CTCL) and graft-versus-host disease, involves *ex vivo* treatment of isolated leukocytes of a patient with the photosensitizing drug 8-methoxypsoralen (8-MOP) and ultraviolet-A (UV-A) exposure before reinfusion back to the patient. However, 8-MOP binds to both diseased and normal cells and thus kills both types of the cells after UV-A illumination with little selectivity. Clinically, this modality gives only partial response in the majority of treated patients. 5-Aminolevulinic acid (5-ALA), a precursor of the potent photosensitizer protoporphyrin IX (PpIX), has been shown to selectively induce PpIX in activated T lymphocytes (T cells) and could be an alternative for 8-MOP. The objectives of this study were to investigate *ex vivo* 5-ALA dark toxicity, 5-ALA-induced PpIX production and photodynamic effect on T cells obtained from clinical ECP patients after the treatment of 5-ALA or 8-MOP plus a built-in certified UV-A source in the commercial Therakos^{TM} Photopheresis System.

Materials and Methods: Flow cytometry was used to study dark cytotoxic effects of 5-ALA on human leukocytes, to measure the production of 5-ALA-induced PpIX in $CD25^+$ activated T cells from both diluted mononuclear cells and undiluted buffy coat samples of ECP patients and to compare photodynamic effects on $CD4^+$, $CD8^+$ and $CD25^+$ T cells with 5-ALA/UV-A or 8-MOP/UV-A.

Results: No dark toxicity of 5-ALA on the leukocytes of ECP patients was seen at concentrations up to 10 mM for an incubation of up to 20 hours. 5-ALA-induced PpIX was produced more in $CD25^+$ activated T cells than resting T cells in both diluted mononuclear cells and undiluted buffy coat samples, although there was a huge variation of samples from different individual patients. The $CD4^+$, $CD8^+$ and $CD25^+$ T cells treated with 5-ALA/UV-A were killed more than those treated with 8-MOP/UV-A.

Conclusion: These results suggest that 5-ALA/UV-A may have the potential for improving the efficacy of ECP.

Keywords

Extracorporeal photopheresis; 8-methoxypsoralen; 5-aminolevulinic acid; protoporphyrin IX; cutaneous T-cell lymphoma; graft-versus-host disease

INTRODUTION

The extracorporeal photopheresis (ECP) technology was initially approved by the U.S. Food and Drug Administration (FDA) 30 years ago for the treatment of cutaneous T-cell lymphoma (CTCL) [1]. Recently, a number of T-cell-mediated diseases are being explored, including graft-versus-host disease (GvHD) [2]. CTCL is a heterogenous group of non-Hodgkin's lymphoma with erythroderma and circulating tumor cells [3]; while GvHD is a serious immunologically-mediated complication of allogeneic hematopoietic cell transplantation in the host due to the incompatible genetic profiles of the donor and host with significant morbidity and mortality [4].

ECP is a combination of apheresis with photochemotherapy in which white blood cells (buffy coat) are initially separated from the whole blood and then exposed to the photosensitizing compound, 8-methoxysoralen (8-MOP) and ultraviolet-A (UV-A) light before being re-infused back to the patient [1, 2]. ECP is used for the treatment of CTCL and GvHD in Europe and North America [2, 5], but the exact action mechanism of this modality is not fully understood. The current hypothesis is that cell-specific proteins released by ECP-mediated apoptotic CTCL cells are taken up by dendritic cells *ex vivo* to induce a specific response eliminating residual untreated CTCL cells *in vivo* [2, 6, 7]. In GvHD, it is believed that ECP may have an inhibitory effect on the immune system without major side-effects, possibly through altered T-cell functions and modulation of dendritic cell (DC) maturation [2, 6, 7].

However, the major drawback of this therapy is that 8-MOP binds to DNA of both diseased and normal cells, together with carcinogenic UV-A illumination, to potentially induce these healthy bystander cells to be mutated or malignant. For example, PUVA, a standard photochemotherapy using a psoralen and UV-A for psoriasis, has been well documented to induce the mutagenic effects on human skin and the resulting carcinogenesis [8]. It is thus likely that similar types of mutations may occur during 8-MOP-ECP. In addition, it kills both diseased and normal cells with little selectivity. Further, the mechanism of its action is unclear, so that it makes it difficult to improve efficacy of this therapy. Clinically, this modality extends over a long period of time and gives only partial response in the majority of treated patients. There is thus a need for a non-toxic, selective, short duration and more effective alternative.

Photodynamic therapy (PDT) typically involves systemic or topical administration of a lesion-localizing photosensitizer and its subsequent activation by visible light. This results

primarily in a singlet oxygen-induced photodamage to the lesion [9]. Results from preclinical and clinical studies conducted worldwide over a 40-year period have established PDT as a clinical treatment modality of a number of malignant and non-malignant disorders [9]. PDT with chemically synthesized sensitizers, however, has a major side-effect of skin phototoxicity, limiting clinical PDT to a great extent. Considerable interest has been directed towards developing a new PDT regimen that relies on an endogenously synthesized sensitizer [10, 11]. In the first step of the heme biosynthetic pathway 5-aminolevulinic acid (5-ALA) is formed from glycine and succinyl CoA. The last step is the incorporation of iron into protoporphyrin IX (PpIX, a potent photosensitizer) and takes place in the mitochondria under the action of the rate-limiting enzyme, ferrochelatase. By adding exogenous 5-ALA, the naturally occurring PpIX, together with minor amounts of some other tetrapyrroles [12], may accumulate because of the limited capacity and/or low activity of ferrochelatase. Porphobilinogen (PBG) deaminase is another enzyme of the heme synthesis pathway. Its activity is higher in hyper-proliferative cells; while that of ferrochelatase is lower, so that PpIX accumulates with a high degree of selectivity in these cells [10, 11]. Such selectivity may also be due to the fact that actively dividing cells overuse intracellular iron stores for their cytochrome and DNA synthesis and thus do not convert PpIX into heme efficiently. This intracellular PpIX accumulation has initially been reported by Malik et al. in blood malignant cell lines *in vitro* [13, 14] and later exploited for its application in photodiagnosis and PDT [10, 11]. Clinically, topically applied 5-ALA-PDT is already an approved treatment modality for actinic keratosis and basal cell carcinoma of the skin [15]; while oral administration of 5-ALA is employed for the photodetection of glioma [16]. .

Our previous studies have established a broad biological basis for possibly improving photopheresis technology with porphyrin precursors and non-carcinogenic visible light illumination [17-20]. 5-ALA could, for example, be advantageous in ECP because 5-ALA-PDT induces cell death in transformed/activated lymphocytes selectively due to an enhanced production of PpIX from 5-ALA in these cells.

The aims of this study were to investigate *ex vivo* 5-ALA dark toxicity, 5-ALA-induced PpIX production and photodynamic effects with 5-ALA/UV-A and 8-MOP/UV-A on activated T cells obtained from clinical ECP patients with CTCL or GvHD, in preparation for future clinical trials with 5-ALA-based ECP.

MATERIALS AND METHODS

Cell Culture and Reagents

Cells were cultured in 10% heat-inactivated fetal calf serum (FCS) in the RPMI-1640 growth medium (Gibco, Grand Island, NY, USA). 5-Aminolevulinic acid (5-ALA) (Sigma Aldrich, St Louis, MO, USA) was dissolved in PBS or physiological saline to a concentration of 100 mM and immediately diluted further in wells. The stock solution of 8-methoxypsoralen (8-MOP) (Sigma Aldrich) was made in absolute ethanol (4.62 mM) and the stock solution of phytohemagglutinin (PHA) (Sigma Aldrich) was diluted in sterile PBS (1 mg/mL), and both were kept at -20 ^{o}C until use.

Samples from Patients and Healthy Donors

During a standard ECP less than 15% of the total blood volume is taken from the patient using an intravenous line [21]. The white blood cells are separated from red blood cells and plasma and treated with 8-MOP and UV-A before they are returned to the patient. In this study CTCL and chronic GvHD patients treated with ECP at the National Photopheresis Centre, St. Olav's University Hospital, Trondheim, Norway, were selected. A small volume of up to 5 mL white blood cells (buffy coat) was removed from the collecting bag using a sterile syringe, a volume equivalent to about 2-3% of the total volume collected for an ECP treatment. All patients gave informed consent to participate. Buffy coats from healthy blood donors were from the Blood Bank, St. Olav's University Hospital. The study was approved by the regional committee for medical research ethics (REK approvals of # 2009/2245 and 2013/134).

Cell Isolation

Isolation of mononuclear cells (MNC) from buffy coat was done according to the following procedure: Buffy coats were layered over 5 mL of Lymphoprep (Axis-Shield, Oslo, Norway) in 15 mL tubes at room temperature. The tubes were centrifuged at 800 g for 30 min. The mid layer was transferred to a fresh tube and washed twice in RPMI medium containing 1% FCS by centrifugation at 100 g for 10 min for the removal of excess platelets. Cells were then re-suspended in growth medium, counted and used in downstream experiments.

Flow Cytometry Analyses

Cells were kept on ice and protected from light during the labeling protocol. In general, cells were transferred to flow tubes, washed with cold PBS and treated with ACK lysis solution (Thermo Fisher Scientific, Waltham, MA, USA) to remove red blood cells prior to analysis when necessary. The cells were then washed twice with PBS and labeled with Fixable Viability Dye eFluor 450 (eBioscience, San Diego, CA, USA). After a 30-min incubation, the cells were washed in PBS/0.1% BSA before another 30-min incubation with conjugated antibodies. The cells were then washed with PBS/0.1% BSA and an Annexin V solution (Alexa Fluor 647 or FITC, depending on the antibody combinations) (Thermo Fisher Scientific) was added. After incubation for 1 hour the cells were analyzed by flow cytometry. For some experiments labeling with the viability dye and Annexin V was not included. Antibodies used to analyze MNC subpopulations by flow cytometry were: CD45 PerCP Cy5.5 (cat. no. 45-0459-42), CD3 PerCP Cy5.5 (cat. no. 45-0037-42), CD3 APC (cat. no. 17-0037-42), CD4 FITC (cat. no. 11-0049-42), CD8 FITC (cat. no. 11-0088-42), CD19 FITC (cat. no. 11-0199-42) (all eBioscience), and CD25 FITC (cat. no. 356106) (Biolegend, San Diego, CA, USA). CD45 was used as a marker for all types of leukocytes, CD3 for all subsets of T cells, CD4 and CD8 for helper- and cytotoxic-T cells, respectively; CD25 for activated T cells and CD19 for B cells, All flow cytometry analyses were performed on an LSR-II flow cytometer (BD Biosciences, San Jose, CA, USA) with FACS Diva software (BD Biosciences). The plots were analysed with FlowJo V10 (TreeStar, Ashland, OR, USA).

Dark Toxicity

For diluted patient samples 3×10^5 MNC were seeded per well in 96-well plates. 5-ALA or 8-MOP dissolved in PBS or physiological saline was added to a final volume of 100 µL. For undiluted patient samples 90 µL of buffy coat were seeded per well and a 5-ALA or 8-MOP solution was added to a final volume of 100 µL. The cells were then incubated for 20 hours in the dark at 37^oC in a humidified atmosphere with 5% CO_2. Cell viability was measured by flow cytometry using FixableViability Dye eFluor 450 and Annexin V as described above.

***In vitro* Activation of T Cells**

CD3 antibody (eBioscience, cat. no. 16-0037-85) was diluted 1:200 in sterile PBS and used to coat wells in 24-well plates for 1 hour at 37^oC. The antibody solution was then removed and the wells were washed 3 times with PBS. MNC were diluted in growth medium to 3×10^6/mL and 1 mL was added per well. CD28 antibody (eBioscience, cat. no. 16-0289-85) (1 µg/mL) was added to the wells and the plate was incubated for three days before further

experiments. For comparison, some cells were activated with PHA (5 µg/mL) during the same time period.

Intracellular 5-ALA-Induced PpIX

Diluted (3×10^5 resting or activated) MNC and undiluted buffy coats were seeded per well in 96-well plates. The cells were then incubated with 5-ALA at a concentration of 3 mM or 10 mM for 1 hour in the dark at 37^{o}C in a humidified atmosphere with 5% CO_2. Intracellular 5-ALA-induced PpIX was measured with flow cytometry using a 405-nm laser for the excitation and 670 LP and 705/70 BP emission filters.

***Ex vivo* ECP Treatment**

A total of 3×10^5 MNC were seeded per well in 96-well plates. 5-ALA or 8-MOP was added to a final volume of 100 µL as described before. The cells were incubated for 1 hour in the dark. An UV-A source in the UVAR-XTS™ Photopheresis System (Therakos, Wokingham, UK) was used to illuminate the cells. The horizontal positioning of the photoactivation chamber makes it possible to lay whole plates in the machine, resembling the illumination of a patient's buffy coat during a standard ECP procedure. The light intensity (0.53 mW/cm^2) and homogeneity of the UV-A source emitting light mainly in the region of 320-410 nm [20] were controlled with an Ocean Optics spectrometer USB4000 (Dunedin, FL, USA). After illumination the plates were incubated for 1 or 20 hours at 37 ^{o}C before the measurement of survival of $CD4^+$ and $CD8^+$ T cells with flow cytometry.

Statistical analysis

Statistical significance was calculated with unpaired t-test or one-way ANOVA (Tukey's multiple comparison test) in GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

RESULTS and DISCUSSION

Dark Cytotoxicity of 5-ALA

Previous studies have indicated selective production of 5-ALA-induced PpIX in artificially activated T cells as compared to resting counterparts from healthy donors [22], but the present study has, for the first time, explored the possibility of using 5-ALA to photodeplet activated T cells *ex vivo* in the samples of ECP patients in the preparation of a future clinical trial using the standard Therakos™ Photopheresis System. Generally, the time of incubation with 5-ALA at a concentration of 1 mM in cells *in vitro* is 4 hours or longer to produce a therapeutic amount of PpIX. However, the Therakos™ Photopheresis System is designed as a one-go, automatic close system. To be feasible and relevant to a clinical 5-ALA use of this System, only one hour with a higher concentration of 3 or 10 mM of 5-ALA was employed to incubate cells in this study.

The effects of 5-ALA on viability of $CD45^+$ leukocytes (3×10^6/mL) from 6 standard ECP-treated patients were measured after incubation with 5-ALA (10 mM) for 20 hours in the dark. The results are presented as viable cells relative to those of the medium control in each sample (Figure 1A). No dark cytotoxicity of 5-ALA was found under the conditions in the diluted buffy coat samples from the standard ECP patients. To compare the dark cytotoxicity of 8-MOP, the cell viability of diluted $CD4^+$ or $CD8^+$ T cells from 4 ECP patients was measured after incubation with 5-ALA (10 mM) or 8-MOP (1 μM, a clinically used dose). Neither 5-ALA nor 8-MOP showed any dark cytotoxic effects on the two subpopulations of T cells (Figure 1B). Moreover, the dark cytotoxicity on different subsets of leukocytes was studied in the undiluted patient samples (n=5 to 10) with no significant reduction in the cell viability of $CD45^+$, $CD4^+$, $CD8^+$ and $CD19^+$ leukocytes (Figure 1C).

5-ALA-PpIX Production in Activated T Cells

The intracellular fluorescence signals of 5-ALA-induced PpIX were flow cytometrically quantified by the geometric mean fluorescence intensity in this study. Since the efficacy of 5-ALA-PDT depends largely on the cellular ability to produce PpIX during 5-ALA incubation, we started to activate MNC from a healthy donor with anti-CD3/CD28 or PHA, followed by the incubation of 5-ALA (3 mM) for 1 hour. As shown in Figure 2A (histograms) and Figure 2B (geometric mean fluorescence intensities), 5-ALA-induced PpIX was produced much more in activated than resting T cells. Further, the T cells activated with anti-CD3/CD28 produced more 5-ALA-PpIX than those activated with PHA.

CD25 has often been used as a marker for activated T cells. Activation with anti-CD3/CD28 or PHA in this study gave a higher fraction of $CD25^+$ T cells as compared to that of resting T cells (Figure 3A). Moreover, the treatment with anti-CD3/CD28 led to a higher production of $CD25^+$ T cells (80%) than PHA activation (60%) (Figure 3A), a finding in line with the higher levels of 5-ALA-PpIX in the anti-CD3/CD28-treated T cells than the PHA treated T cells (Figures 2A & 2B). Based on these results, CD25 was used as a marker for activated T cells to study 5-ALA-PpIX production in patient samples without prior activation. About 8-20% of $CD3^+/CD25^+$ T cells were found in the buffy coats obtained from 4 patients (Figure 3B). These samples were diluted and incubated with 5-ALA in the dark for one hour at the concentration of 3 or 10 mM. While PpIX was neither seen in $CD3^+/CD25^-$ nor $CD3^+/CD25^+$ cells without 5-ALA incubation; the average PpIX production was higher in $CD3^+/CD25^+$ cells than $CD3^+/CD25^-$ cells at both concentrations of 5-ALA used. A similar amount of PpIX was produced after the ALA incubation with 3 mM or 10 mM (Figure 3C). Moreover, whole buffy coats (without any dilution) from 8 patient samples with 2.5-15% $CD3^+/CD25^+$ activated cells (data not shown) were incubated with 3 or 10 mM 5-ALA in the dark for one hour and similar results were obtained (Figure 3D). It should be pointed out that there was a huge variation of individual samples with respect to PpIX production from 5-ALA, probably due to different diseases and/or various clinical situations of individual patients with CTCL or GvHD.

Photodynamic Effect on T Cells with 5-ALA/UV-A or 8-MOP/UV-A

The built-in certified UV-A source in the standard Therakos™ Photopheresis System with light mainly in the range of 320-410 nm was used to activate 5-ALA-induced PpIX for photodynamic effect, although the main absorption peak of PpIX is at 405 nm. UV-A-mediated photodynamic effects were compared on T cells of patient samples pretreated with 5-ALA or 8-MOP. Diluted MNC in 96-well plates were incubated with 5-ALA (3 mM) or 8-MOP (1 µM) for 1 hour in the dark, followed by the UV-A illumination of 0.159 J/cm^2 in the Therakos UVAR XTS™ Photopheresis System. The viability of $CD4^+$ and $CD8^+$ T cells was then measured at 1 hour (Figure 4A) or 20 hours (Figures 4B & 4C) after illumination. There were no evident changes in the cell survival of $CD4^+$ or $CD8^+$ T cells at 1 hour after treatment (Figure 4A). However, after 20 hours 5-ALA/UV-A killed more $CD4^+$ and $CD8^+$ cells than 8-MOP/UV-A in all respective individual samples of the same patients (Figure 4B). On an average, UV-A alone killed about 40% $CD4^+$ cells and 50% $CD8^+$ cells; respectively (Figure 4C). Only 7% $CD4^+$ and 1% $CD8^+$ cells survived after UV-A in combination with 5-ALA.

With 8-MOP UV-A killed totally about 70% $CD4^+$ and $CD8^+$ cells (Figure 4C). These results indicate that 5-ALA/UV-A may have an advantage over 8-MOP/UV-A to improve the efficacy of ECP and needs further investigations in a larger number of patient samples with similar clinical situations of a single disease. Furthermore, the effectiveness of this modality can be significantly enhanced if a visible light source is used with 405 nm where PpIX has a major absorption peak.

CONCLUSION

In conclusion, no dark toxicity of 5-ALA on leukocytes of ECP patients was seen *ex vivo* at concentrations up to 10 mM for an incubation of 20 hours. PpIX was produced more in $CD25^+$ activated T cells than resting T cells in both diluted MNC and undiluted buffy coats, although there was a huge variation of samples from different individual patients. The $CD4^+$ and $CD8^+$ T cells treated with 5-ALA/UV-A were killed more than those treated with 8-MOP/UV-A. These results suggest that 5-ALA/UV-A may have the potential for improving the efficacy of ECP.

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Figure legends

Figure 1. Dark toxicity of 5-ALA on diluted MNC and undiluted buffy coats

Mononuclear cells (MNC) were incubated with 0 or 10 mM 5-ALA for 20 hours in the dark. Cells labelled with an anti-CD45 antibody as a leukocyte marker, Fixable Viability Dye for dead cells and Annexin V for apoptotic cells were measured by flow cytometry. The cells that were negative for both the Viability Dye and Annexin V were considered as viable cells and plotted as percentage of the medium control (without 5-ALA) (A). Further, the dark cytotoxicity of $CD4^+$ or $CD8^+$ T cells was compared between 5-ALA (10 mM) and 8-MOP (1 µM) (B). Undiluted patient buffy coat samples were incubated with 0 or 10 mM 5-ALA for 20 hours in the dark before analyzing the viability of various subpopulations of leukocytes (C). A & B show duplicate measurements and error bars represent the variation between the two (+/- 1 SD). (C) presents single measurements with various numbers of patient samples for each subpopulation of leukocytes as indicated. There were no significant differences in cell viability between groups (unpaired t-test, $P>0.05$).

Figure 2. 5-ALA-induced PpIX in activated $CD3^+$ T cells

Healthy donor MNC were activated *in vitro* with anti-CD3/CD28 or PHA for 3 days. The cells were incubated with 5-ALA (3 mM) for 1 hour and analyzed for the PpIX content in $CD3^+$ T cells. The PpIX levels in resting (unactivated) cells without 5-ALA, 5-ALA-treated resting cells, anti-CD3/CD28-activated- and PHA-activated cells are shown (A). (B) shows the geometric mean PpIX fluorescence intensity in the same samples as (A). The number in B is an average of two repeats and the error bars indicate the variation between the two (+/- 1 SD).

Figure 3. 5-ALA-induced PpIX in $CD25^+$ T cells

The percentages of $CD25^+$ activated T cells were measured in the resting, anti-CD3/CD28-activated and PHA-activated MNC (A) and also in the buffy coat samples of patients (n=4) (B). The PpIX levels were measured in the $CD25^-$ and $CD25^+$ cells of diluted patient buffy coat samples (n=4) (C) and undiluted patient buffy coat samples (n=8) (D) after incubation with 3 mM or 10 mM of 5-ALA for 1 hour in the dark. Asterisks indicate the degree of significance, one-way ANOVA (*, $P\leq0.05$; and **, $P\leq0.01$).

Figure 4. Comparison of photodynamic effects between 5-ALA/UV-A and 8-MOP/UV-A

MNC from 4 patient buffy coat samples were seeded in 96-well plates and left untreated or treated with 5-ALA (3 mM) or 8-MOP (1 µM) for 1 hour in the dark. The plates were then exposed to UV-A at a dose of 0.159 J/cm^2 in the Therakos UVAR XTS^{TM} Photopheresis System. Parallel plates with control cells were kept in the dark. The cells were then labelled with anti-CD4 and CD8 antibodies, Fixable Viability Dye and Annexin V after 1 hour (A) or 20 hours (B & C). (B) shows the survivals of CD4$^+$ cells and CD8+ cells of the same individual patient samples (P5-P8 are the numbers of patient samples) treated with 5-ALA/UV-A or 8-MOP/UV-A; while (C) presents the grouped data from (B). The cell survivals were measured with flow cytometry in the same way as described in Figure 1. Asterisks above bars indicate the degree of significance, one-way ANOVA (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; and ****, $P \leq 0.0001$).

Figure 1

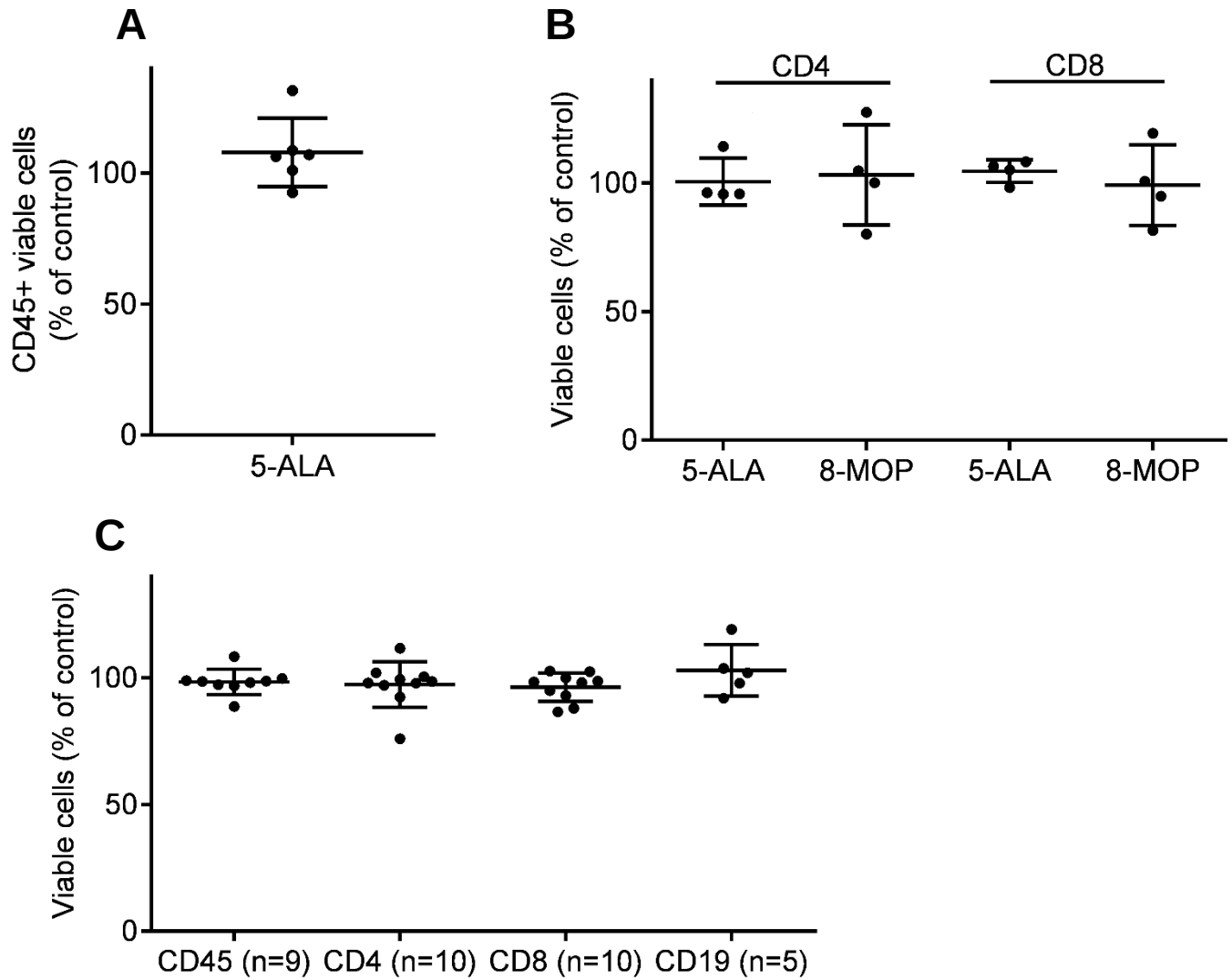
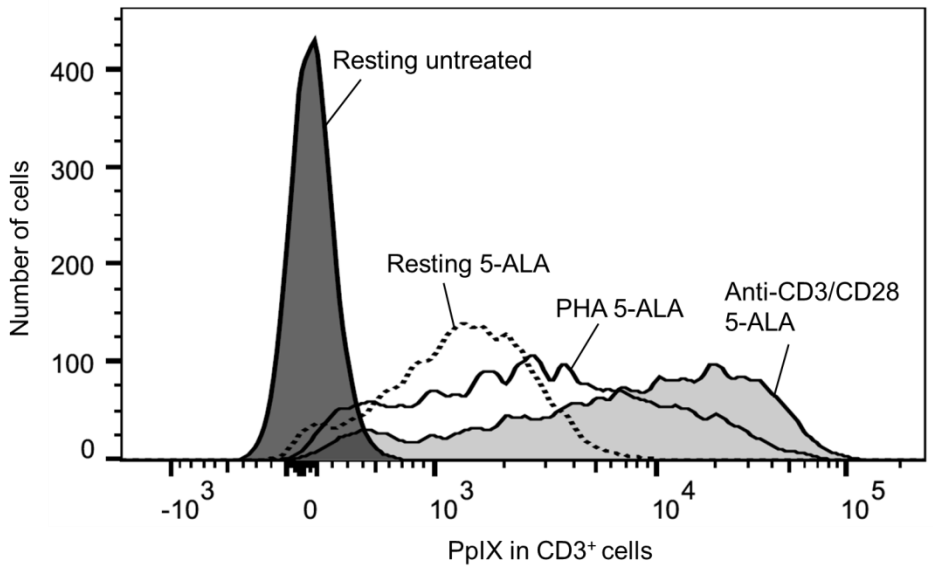

A
CD45+ viable cells (% of control)
100
50
0
5-ALA
B
CD4
CD8
Viable cells (% of control)
100
50
0
5-ALA
8-MOP
5-ALA
8-MOP
C
Viable cells (% of control)
100
50
0
CD45 (n=9)
CD4 (n=10)
CD8 (n=10)
CD19 (n=5)

Figure 2

A


Resting untreated
Resting 5-ALA
PHA 5-ALA
Anti-CD3/CD28
5-ALA
Number of cells
400
300
200
100
0
-10^3
0
10^3
10^4
10^5
PpIX in CD3+ cells

B

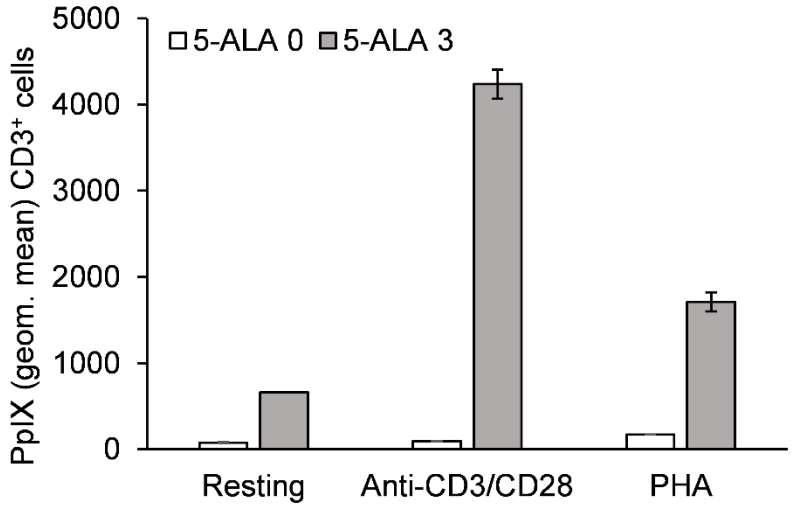

5-ALA 0
5-ALA 3
PpIX (geom. mean) CD3+ cells
5000
4000
3000
2000
1000
0
Resting
Anti-CD3/CD28
PHA

Figure 3

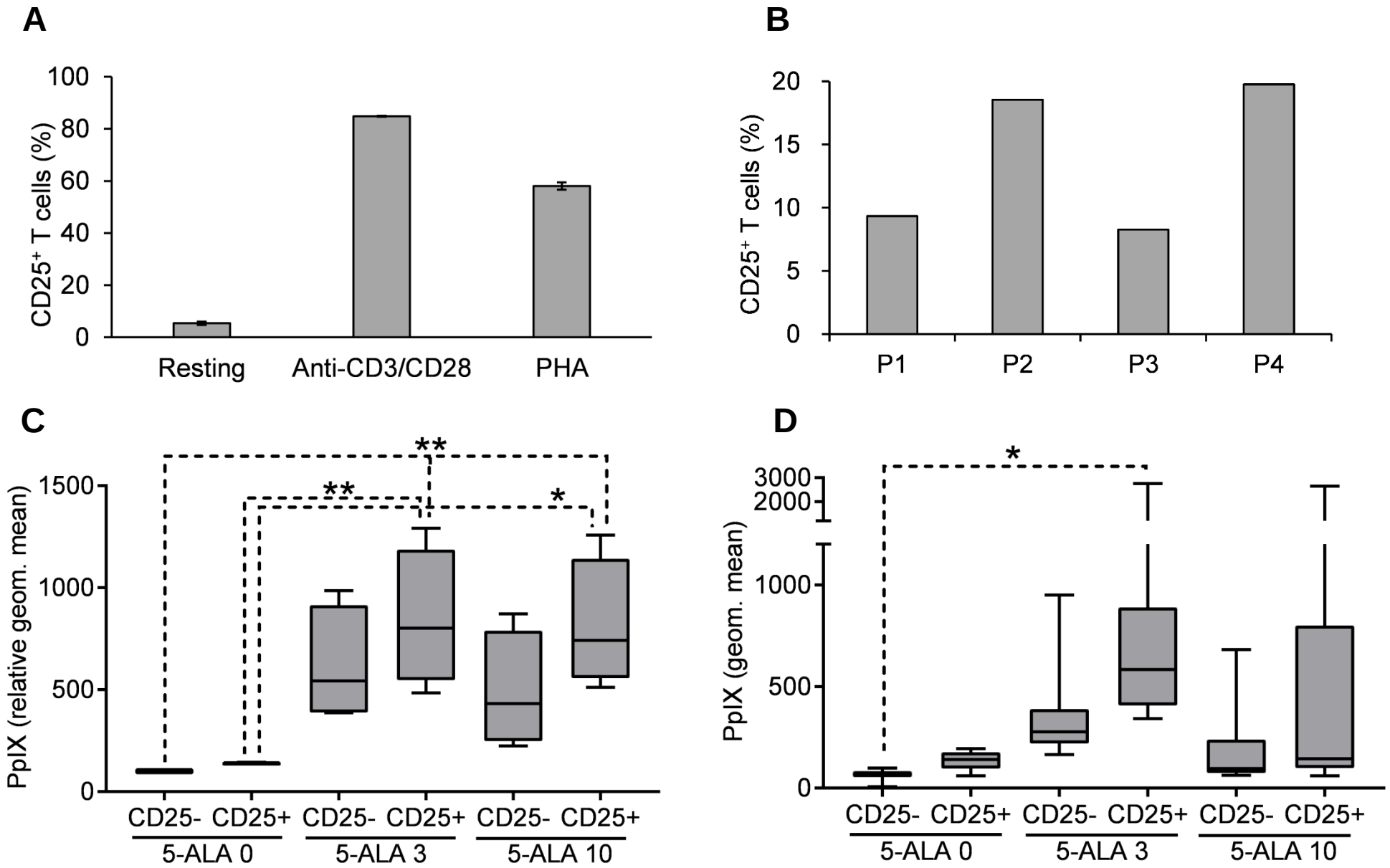

A
100
80
60
40
20
0
CD25+ T cells (%)
Resting
Anti-CD3/CD28
PHA
B
20
15
10
5
0
CD25+ T cells (%)
P1
P2
P3
P4
C
**
**
*
1500
1000
500
0
PpIX (relative geom. mean)
CD25- CD25+ CD25- CD25+ CD25- CD25+
5-ALA 0
5-ALA 3
5-ALA 10
D
*
3000
2000
1000
500
0
PpIX (geom. mean)
CD25- CD25+ CD25- CD25+ CD25- CD25+
5-ALA 0
5-ALA 3
5-ALA 10

Figure 4

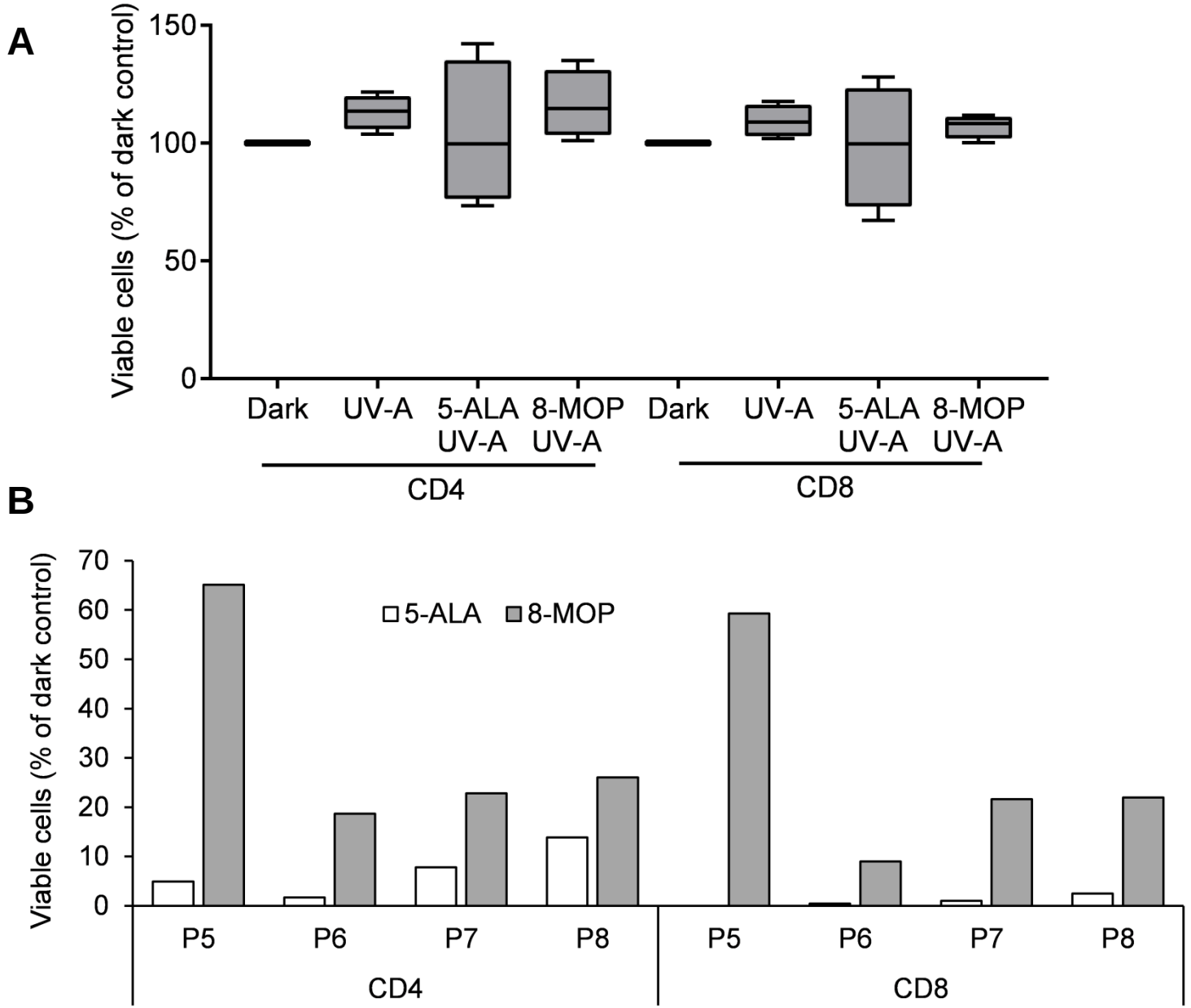
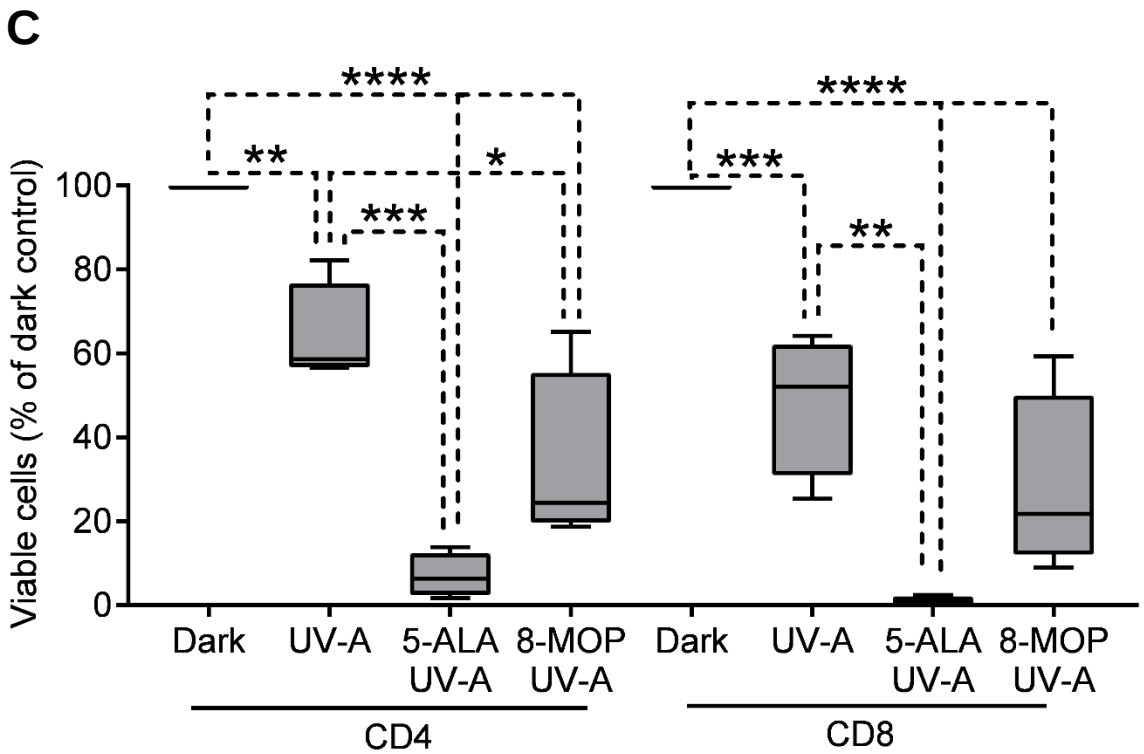

A
Viable cells (% of dark control)
150
100
50
0
Dark
UV-A
5-ALA UV-A
8-MOP UV-A
Dark
UV-A
5-ALA UV-A
8-MOP UV-A
CD4
CD8
B
Viable cells (% of dark control)
70
60
50
40
30
20
10
0
5-ALA
8-MOP
P5
P6
P7
P8
P5
P6
P7
P8
CD4
CD8

Figure 4


C
Viable cells (% of dark control)
100
80
60
40
20
0
****
**
*
***
****
***
**
Dark
UV-A
5-ALA UV-A
8-MOP UV-A
Dark
UV-A
5-ALA UV-A
8-MOP UV-A
CD4
CD8