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**Photopheresis with UV-A light and 8-methoxypsoralen leads to cell
death and to release of blebs with anti-inflammatory phenotype in
activated and non-activated lymphocytes**

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Widmung

Allen,
die mit Rat und Tat zum Gelingen dieser Arbeit
beigetragen haben!

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1 Zusammenfassung

1.1 Hintergrund

Die extrakorporale Photopherese mittels UV-A Licht in Kombination mit 8-Methoxypsoralen (PUVA) wird bei Autoimmunkrankheiten, beim kutanen T-Zell-Lymphom, bei Transplantatabstoßungsreaktionen und auch bei der Graft-versus-Host-Disease therapeutisch genutzt. Allerdings ist der genaue Wirkmechanismus von UV-A-Licht in Kombination mit Psoralen noch unklar. Ziel dieser Arbeit war es deshalb, den Zelltod aktivierter als auch nicht-aktivierter Lymphozyten nach PUVA-Behandlung und die Rate der von den Lymphozyten freigesetzten Blebs sowie deren Antigenstruktur zu untersuchen.

1.2 Material und Methoden

Zum Nachweis von Apoptose und Nekrose wurden die Lymphozyten mit AnnexinV (AxV)-FITC und Propidiumiodid (PI) gefärbt. AxV-FITC wurde verwendet, um die Exposition von Phosphatidylserin (PS) nachzuweisen; PI, um zwischen apoptotischen (AxV+/PI-) und nekrotischen (AxV+/PI+) Zellen zu differenzieren. Für den Nachweis der Zellkerndegradation wurden die Lymphozyten mit PI in Anwesenheit von Triton X-100 gefärbt. Die Messungen erfolgten mittels Durchflusszytometrie. Die Freisetzung von Blebs und die Zusammensetzung ihrer Oberflächenstruktur wurden ebenfalls mittels Durchflusszytometrie analysiert.

1.3 Ergebnisse und Beobachtungen

In Anwesenheit von 8-Methoxypsoralen führte die Bestrahlung mit UV-A-Licht zu einer hochsignifikanten Erhöhung des Zelltodes in der Population der aktivierten Lymphozyten. Der gleiche Effekt, aber in geringerer Ausprägung, konnte in der Population der nicht-aktivierten Lymphozyten beobachtet werden. Die höchste Rate von an der Oberfläche exprimiertem Phosphatidylserin nach PUVA-Behandlung zeigten die Blebs der aktivierten Lymphozyten. Diese Blebs wiesen zudem eine hohe Expressionsrate der Antigene CD5 und CD8 sowie eine niedrige Rate an CD28 und CD86 auf.

1.4 Schlussfolgerung

PUVA-Behandlung übt durch Apoptoseinduktion und die damit einhergehende Sekretion von apoptotischen Blebs mit immunsuppressiver Oberflächenstruktur einen anti-inflammatorischen Effekt aus.

2 Summary

2.1 Background

Extracorporeal photopheresis is a therapy for treatment of autoimmune diseases, cutaneous T-cell lymphoma, organ graft rejection as well as graft-versus-host-diseases. The exact mechanism how the combination of 8-methoxypsoralen plus UV-A irradiation (PUVA) acts is still unclear. We investigated the cell death of activated and non-activated lymphocytes after PUVA treatment as well as the rate of released blebs and their antigen composition.

2.2 Material and Methods

For the detection of apoptosis and necrosis, the lymphocytes were stained with annexinV (AxV)-FITC and propidium iodide (PI). AxV-FITC was used to identify phosphatidylserine exposure, and PI to distinguish between apoptotic (AxV+/PI-) and necrotic (AxV+/PI+) cells. To monitor nuclear degradation, the cells were stained with PI in the presence of the detergent Triton X-100. The release of blebs and their surface composition was also analysed by flow cytometry.

2.3 Results

In presence of 8-MOP, UV-A light highly significantly increased the cell death of activated lymphocytes. The same was observed to a lesser extent in non-activated cells. Blebs derived from activated lymphocytes after PUVA treatment showed the highest surface exposition of phosphatidylserine. These blebs also displayed a high exposure of the antigens CD5 and CD8 as well as a low exposure of CD28 and CD86.

2.4 Conclusion

PUVA treatment exerts anti-inflammatory effects by inducing apoptosis and apoptotic cell-derived blebs with immune suppressive surface composition.

3 Einleitung

Die extrakorporale Photopherese (ECP) wird zur Therapie verschiedener Autoimmunkrankheiten, des kutanen T-Zell-Lymphoms, bei Transplantatabstoßungsreaktionen sowie bei der Graft-versus-Host-Disease eingesetzt. Hierfür werden periphere mononukleäre Blutzellen des Patienten durch Apherese gesammelt, außerhalb des Körpers unter Zugabe von 8-Methoxypsoralen (8-MOP) mit UV-A-Licht (PUVA) bestrahlt und anschließend reinfundiert. Das photoaktive Psoralen bindet an Pyrimidinbasen und interkaliert in die DNA [11]. Es wird vermutet, dass die ECP immunsuppressiv wirkt; allerdings ist das Wissen über den genauen Wirkmechanismus aktuell noch sehr bruchstückhaft. Es wurde schon gezeigt, dass die Behandlung aktivierter Zellen mit PUVA nach einiger Zeit zum Zelltod führt [16] und zudem die Bildung dendritischer Zellen (DZ) mit tolerogenem Phänotyp induziert [17].

Apoptose und primäre Nekrose sind die extremen Arten des Zelltods [15], wobei apoptotische Zellen gewöhnlich nicht- oder sogar anti-entzündlich wirken und so zur immunsuppressiven Wirkung beitragen [26]. Im Gegensatz dazu führt Nekrose in der Regel zu Entzündungsreaktionen und zu einer Aktivierung des Immunsystems [2]. Apoptose wurde als generalisierter Mechanismus zur Entfernung unerwünschter Zellen aus dem Immunsystem identifiziert und ist durch spezifische morphologische Veränderungen der absterbenden Zellen charakterisiert, wohingegen Nekrose durch irreversibles Anschwellen der Zelle bis hin zur Ruptur der Zellmembran gekennzeichnet ist. Die Exposition von Phosphatidylserin (PS) an der Oberfläche apoptotischer Zellen ist ein bedeutender Auslöser für Makrophagen, diese Zellen auf nicht- oder anti-entzündlichem Weg zu entfernen [1]. Zudem sezernieren apoptotische Zellen abhängig vom Stimulus auch Mikropartikel und apoptotische Blebs. Wie bereits bei Schiller et. al. beschrieben, wird der Begriff "Blebs" in dieser Arbeit für sub-zelluläre Partikel verwendet, die sich von apoptotischen Zellen abgeschnürt haben [24]. Diese Blebs können, abhängig von der PS-Expression an ihrer Oberfläche, auf Regulatoren der Immunantwort, als auch auf Proteine aus dem Zytosol, dem

endoplasmatischen Retikulum und dem Zellkern modulatorisch wirken [7]. Bis jetzt wurde allerdings weder die Freisetzung von Blebs nach PUVA-Behandlung untersucht, noch deren Antigenstruktur charakterisiert.

Deshalb wurde im Rahmen dieser Arbeit jeweils die Rate an Apoptose, Nekrose, sowie die Anzahl der freigesetzten Blebs in aktivierten und nicht-aktivierten Lymphozytenpopulationen nach Behandlung mit UV-A-Licht, mit photoaktivem 8-MOP oder PUVA untersucht. Zusätzlich wurde die Exposition von immunmodulatorischen Oberflächenmolekülen auf Blebs nach PUVA-Behandlung analysiert.

4 Introduction

Extracorporeal photopheresis (ECP) is a therapy used to treat various autoimmune diseases, cutaneous T-cell lymphoma, organ graft rejection as well as graft-versus-host-diseases. For ECP, peripheral blood mononuclear cells are collected by apheresis, extracorporeally irradiated with UV-A light in presence of 8-Methoxypsoralen (8-MOP) and re-infused into the patient. The photoactive 8-MOP binds to pyrimidin bases and intercalates into DNA [11]. ECP has been suggested to be immune suppressive. The knowledge on the exact mechanism how the combination of UV-A plus 8-MOP (PUVA) acts is still fragmentary. It was previously shown that PUVA treatment leads to cell death in activated cells at later time points after treatment [16]. Furthermore, PUVA induced dendritic cells (DC) with tolerogenic phenotype [17].

Apoptosis and primary necrosis are the extreme kinds of cell death [15]. Apoptotic cells are usually non- or even anti-inflammatory and contribute to immune suppression [26]. In contrast, necrosis normally leads to inflammation and immune activation [2]. Apoptosis was identified to be a general mechanism for removal of unwanted cells from the immune system characterized by specific morphological changes of the dying cells whereas necrosis was characterized by irreversible swelling of cells leading to the disruption of the cell membrane. The surface exposure of phosphatidylserine (PS) on apoptotic cells is a major trigger for the cells to be removed by macrophages in a non- or even anti-inflammatory way [1]. Depending on the stimulus, apoptotic cells also release microparticles and apoptotic blebs. As described by Schiller et al., we used the term blebs for sub-cellular particles that have separated from apoptosing cells [24]. Blebs can be modulators of regulators of immune responses, depending on the expression on their surface of PS, as well as of proteins derived from the cytosol, the endoplasmic reticulum, and the nucleus [7]. By now, the release of blebs under treatment with PUVA and their antigenic characterization are not investigated.

We examined the rate of apoptosis, necrosis and the amount of released blebs of activated and non-activated lymphocytes treated with UV-A light, with the photosensitizer 8-MOP or a combination of both (PUVA). Furthermore, the exposure of immune modulatory surface molecules on blebs after PUVA treatment was analysed.

5 Veröffentlichung

Photopheresis with UV-A light and 8-methoxypsoralen leads to cell death and to release of blebs with anti-inflammatory phenotype in activated and non-activated lymphocytes

5.1 Abstract

5.1.1 Background

Extracorporeal photopheresis is a therapy for treatment of autoimmune diseases, cutaneous T-cell lymphoma, organ graft rejection as well as graft-versus-host-diseases. The exact mechanism how the combination of 8-methoxypsoralen plus UV-A irradiation (PUVA) acts is still unclear. We investigated the cell death of activated and non-activated lymphocytes after PUVA treatment as well as the rate of released blebs and their antigen composition.

5.1.2 Results

In presence of 8-MOP, UV-A light highly significantly increased the cell death of activated lymphocytes. The same was observed to a lesser extent in non-activated cells. Blebs derived from activated lymphocytes after PUVA treatment showed the highest surface exposition of phosphatidylserine. These blebs also displayed a high exposure of the antigens CD5 and CD8 as well as a low exposure of CD28 and CD86.

5.1.3 Conclusion

PUVA treatment exerts anti-inflammatory effects by inducing apoptosis and apoptotic cell-derived blebs with immune suppressive surface composition.

5.2 Introduction

Extracorporeal photopheresis (ECP) is a therapy used to treat various autoimmune diseases, cutaneous T-cell lymphoma, organ graft rejection as well as graft-versus-host-diseases. For ECP, peripheral blood mononuclear cells are collected by apheresis, extracorporeally irradiated with UV-A light in presence of 8-Methoxypsoralen (8-MOP) and re-infused into the patient. The photoactive 8-MOP binds to pyrimidin bases and intercalates into DNA [11]. ECP has been suggested to be immune suppressive. The knowledge on the exact mechanism how the combination of UV-A plus 8-MOP (PUVA) acts is still fragmentary. It was previously shown that PUVA treatment leads to cell death in activated cells at later time points after treatment [16]. Furthermore, PUVA induced dendritic cells (DC) with tolerogenic phenotype [17].

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We examined the rate of apoptosis, necrosis and the amount of released blebs of activated and non-activated lymphocytes treated with UV-A light, with the photosensitizer 8-MOP or a combination of both (PUVA). Furthermore, the exposure of immune modulatory surface molecules on blebs after PUVA treatment was analysed.

5.3 Material and Methods

5.3.1 Reagents

Propidium iodide (PI) was purchased from Sigma-Aldrich (Munich, Germany) and FITC-labelled annexinV (AxV) from responsif GmbH (Erlangen, Germany). Fluorescein isothiocyanate (FITC)-labelled CD8 antibody as well as the Isotype FITC-PE antibody were purchased from Beckman Coulter (Krefeld, Germany), FITC-labelled CD86 antibody from Southern Biotech (Birmingham, USA), and Phycoerythrin (PE)-labelled CD5 and CD28 antibodies from BD Biosciences (Erembodegem, Belgium).

5.3.2 Cells and blebs

Human peripheral blood mononuclear cells (PBMC) were isolated from blood of healthy donors by Ficoll density gradient centrifugation (Ficoll Lymphoflot, Biotest, Dreieich, Germany). Remaining platelets were removed by centrifugation through fetal bovine serum (FBS; Invitrogen, Paisley, UK). The isolated cells were cultured in RPMI medium supplemented with 10% fetal FBS (Biochrom AG, Germany), 1% sodium pyruvate, 2mM glutamine, 100U/ml of penicillin and 100µg/ml of streptomycin (R10 medium). Half of the cells were activated with phytohemagglutinine (PHA, 1 µg/ml, Sigma-Aldrich, Munich, Germany) and Interleukin-2 (IL-2, 10U/ml, Novartis Germany, Nuremberg, Germany) and cultured for 6 days in a humidified atmosphere with 5% CO₂ at 37°C. Non-stimulated cells were cultured shortly in R10 medium and treated immediately. Blebs were identified and quantified with flow cytometry as previously described by Schiller et al. [24].

5.3.3 PUVA treatment

Cell suspensions were irradiated with a UV-A light box (Therakos, West Chester, PA, USA) emitting UV-A light with 18 mW/cm². The cells were irradiated with UV-A for 60 seconds and/or treated with Psoralen [2µg/ml] (8-MOP, UVA-DEX, Ben Venue Laboratories, Bedford, USA). 8-MOP was added to the cell suspension before irradiation with UV-A light. After treatment the cells were incubated up to 48 hours at 37°C at 5% CO₂.

5.3.4 Analyses of apoptosis and necrosis

The cells were stained with annexin V (AxV)-FITC and PI as described previously [14]. Shortly, AxV-FITC was used to identify PS exposure, and PI to distinguish between apoptotic (AxV+/PI-) and necrotic (AxV+/PI+) cells. Cells were incubated for 30 min at 4°C with FITC labelled AxV (5µg/ml) and PI (200ng/ml) suspended in Ringers' solution. Afterwards, the cells were analysed by flow cytometry. To monitor nuclear degradation, the cells were stained with PI in the presence of the detergent Triton X-100 [20]. The cells were incubated for 18 hours at 4°C in the dark and afterwards analysed by flow cytometry.

5.3.5 Staining of blebs with antibodies

Cell suspensions containing blebs were incubated for 30 min at 4°C with FITC labelled antibodies against CD8, CD86 or with PE-labelled antibodies against CD5 or CD28 (each 1µg antibody/100µl cell suspension containing 100.000 cells). In parallel, staining with control antibody was performed. After incubation, the cells were suspended in 400µl of PBS and analysed by flow cytometry.

5.3.6 Analysis by flow cytometry

Flow cytometry was performed with an EPICS XLTM flow cytometer (Beckman Coulter, Hialeah, FL, USA). Excitation was at 488nm, the FITC fluorescence was recorded on a fluorescence 1 (FL1) sensor and the PI fluorescence was recorded on a fluorescence 4 (FL4) sensor. Data analysis was performed with Coulter XLTM software, version 3.

5.3.7 Statistical analysis

At least two independent experiments, each carried out in triplicates, were performed. The two tailed, unpaired Student's t test was used for statistical analyses. Results are displayed as mean + standard deviation (SD).

5.4 Results

8-MOP increases the UV-A light induced cell death of activated lymphocytes

The exposure of PS (Fig. 1A-C) and the nuclear degradation (Fig. 1D-F) of the activated cells was analysed 12 hours after the indicated treatment. The amount of apoptotic cells (AxV pos./PI neg.) after PUVA treatment (Fig. 1C) as well as that of necrotic ones (AxV pos./PI pos.) was significantly higher than after treatment with 8-MOP only (Fig. 1A) or UV-A light (Fig. 1B). Analyses of the nuclear degradation revealed that also the amount of cells with degraded DNA (early and late apoptotic cells) was synergistically increased after PUVA treatment (Fig. 1F) in comparison to 8-MOP (Fig. 1D) or UV-A treated cells (79% in comparison to 19% or 36%, respectively).

Next, we analysed the kinetic of apoptosis and necrosis of activated lymphocytes (Fig. 1G and H). When cells were cultured for up to 48h after PUVA treatment, the rate of apoptotic cells reached almost 60% while the other treatments led to significantly lower amounts of apoptotic cells at all time points investigated (Fig. 1G). As displayed in Fig. 1H, the amount of necrotic cells reached a maximum of about 45% after PUVA treatment. In contrast, very little rates of apoptosis (10 to 15%) and necrosis (5 to 10%) were observed in the untreated control or 8-MOP only treated cells (Fig. 1G and H). Since the stimulated lymphocytes reached a plateau for apoptosis and necrosis already 24h after treatment we focussed our further analyses on the time points 6h, 12h and 24h after treatment.

PUVA treatment increases the amount of apoptosis in stimulated and non-stimulated lymphocytes

The amount of apoptotic activated lymphocytes (approximately 40%) after PUVA treatment was significantly higher compared to non-activated cells (approximately 20%; Fig. 2). In all cases, a higher rate of apoptosis was observed after combined treatment in comparison to single treatment. In contrast to the stimulated cells, non-stimulated ones showed a maximum amount of apoptotic cells of 10 to 20% (Fig. 2A). A similar tendency could be detected for necrotic cells (data not shown). As depicted in Fig. 2B, the nuclear degradation was highest in the case of the treatment of stimulated lymphocytes with PUVA and reached almost 90%. In contrast, stimulated UV-A treated lymphocytes reached a maximum of 50% and stimulated 8-MOP treated cells of about 25%. Again we have for the first time seen an effect of PUVA on non-stimulated cells. However, the stimulation before treatment revealed an about 4 times stronger effect on the degradation of nuclear DNA of the cells.

8-MOP, UV-A and PUVA increase the release of blebs of stimulated and non-stimulated lymphocytes

The amount of released blebs differed strongly between stimulated and non-stimulated lymphocytes after treatment with 8-MOP and/or UV-A. As shown in Fig. 3, stimulated lymphocytes (Fig. 3B) showed a significant higher secretion rate of blebs in comparison to non-stimulated cells (Fig. 3A). After PUVA treatment stimulated cells release about twice of blebs than after UV-A treatment. Notably, 8-MOP only treatment also resulted in a slight but significant increase of blebs. In the stimulated situation, the amount of blebs after the respective treatment differed highly significantly at all time points. Without stimulation, PUVA treated cells also differed highly significantly from 8-MOP only or UV-A treated cells at all time points. 24 hours after treatment all values differed highly significantly. However, there was no significant difference between UV-A and 8-MOP treated cells in the release of blebs until 12 hours after treatment.

Blebs derived from stimulated lymphocytes after PUVA treatment have the highest amount of PS on their surface

The exposure of PS on blebs (AxV positive blebs) could be detected in non-stimulated and stimulated ones. However, the PS exposure was much more pronounced on blebs of stimulated cells (Fig. 4A). The highest amount of PS on blebs could be seen 12 hours after treatment of activated cells with PUVA. 8-MOP only treatment of activated cells led to a very low exposure of PS, comparable to that of blebs derived from non-stimulated cells (Fig. 4A).

Blebs derived from stimulated lymphocytes after PUVA treatment display a distinct surface antigen composition

The analysis of the surface antigen composition of the blebs released by apoptotic activated lymphocytes after PUVA treatment revealed a significantly increase in CD5 expression (max. about 70%) already 6h after treatment (Fig. 4B). Furthermore, 6h after the treatment with PUVA or UV-A, the released blebs expose significantly more CD8 on their surface (max. about 55 %; Fig. 4C) in comparison to 8-MOP treated cells. The exposure of CD5 and CD8 on the blebs differed significantly at all time points of investigation between the different treatments in the case of stimulated cells. In contrast, non-stimulated lymphocytes display no significant change in surface expression of CD8 on blebs after treatment (Fig. 4C). The expression of CD5 was low, but significantly increased 12h and 24h after treatment with PUVA (Fig. 4B). At the beginning of the observation period, blebs of stimulated cells showed a higher CD28 expression in comparison to non-stimulated cells. However, PUVA treatment, UV-A and 8-MOP decreased the expression of CD28 (Fig. 4D). The expression of CD28 on blebs of non-stimulated cells was higher than on stimulated ones 24h after treatment with UV-A or PUVA. Furthermore, the surface exposure of CD86 (Fig. 4E) was higher on blebs of non-stimulated compared to stimulated lymphocytes during the whole observation period. Blebs derived from stimulated cells show a significantly decreased expression of CD86 as early as 12h after treatment with PUVA or UV-A.

5.5 Discussion

In the current study we investigated the rate and form of cell death of activated and non-activated lymphocytes after treatment with 8-MOP, UV-A or PUVA. The activity of caspase-3 and DNA ladder formation increases after UV-A irradiation leading to cellular death [25]. It was previously shown that intravenous injection of PUVA treated cells exerts silencing of the immune system by inducing regulatory T cells [19]. The anti-inflammatory cytokine IL-10 was identified as critical inhibitory component in the sensitization and effector phase of contact hypersensitivity [18]. We showed that after PUVA treatment, lymphocytes die by apoptosis and necrosis. A mixture of an anti-inflammatory (apoptotic cells) and inflammatory milieu (necrotic cells) might therefore be generated *in vivo*. However, the necrotic cells also possessed degraded DNA (Fig. 1F), a hallmark of apoptosis. We conclude that these cells underwent apoptosis before they lost their membrane integrity and became secondarily necrotic. Secondary necrosis might only happen *in vitro*, since apoptotic cells are normally swiftly cleared by macrophages without inducing inflammation or even in an anti-inflammatory context [8]. ECP is used since 20 years in the treatment of chronic graft-versus-host disease (cGVHD). Inflammation often results from the presence of autoantibodies in those diseases [23]. Clearance of apoptotic cells in a an anti-inflammatory way may be an important natural reminder of ongoing tolerance (summarized in [22]). We could show for the first time that as early as 12 hours after PUVA treatment most of the activated and non-activated lymphocytes have already reached their maximum in cell death. Furthermore, the exposure of PS on blebs derived from activated lymphocytes after treatment with PUVA was the highest at this time point. During apoptosis cellular constituents are packed in membrane coated vesicles, in the so called apoptotic blebs. The latter are rapidly engulfed by environmental phagocytes [24]. Apoptotic cells and apoptotic cell-derived blebs acquire distinct surface determinants like PS for specific recognition by responder phagocytes leading to their anti-inflammatory clearance [3]. Consequently, disturbance of the PS-dependent removal results in immune activation [4]. Taken together, blebs are important

immune modulators [21] and are able to reduce the stimulatory capacity of distinct immune cells like macrophages [13]. Our experiments revealed that the phospholipid PS was dominantly exposed by blebs of activated lymphocytes after PUVA treatment. Since PS exerts anti-inflammatory effects, those blebs may serve as trigger for certain tolerance mechanisms, and consecutively be of therapeutic use in GVHD.

We further analysed the surface expression of CD5, CD8, CD28, and CD86, which are known to be regulators of inflammation. Increased expression of CD5 on B cells or T cells protects against autoimmunity. Furthermore, CD5-positive cells may also provide cytokines such as IL-10. In cancer, CD5 expression renders lymphocytes tolerant and unable to recognize and eliminate malignant cells [6]. Our experiments revealed that PUVA treatment of activated lymphocytes results in the generation of many CD5 positive blebs which may contribute to immune suppression. Also the amount of CD8 positive blebs increased after PUVA treatment suggesting that mainly blebs derived from T cells are involved in the immune regulatory mechanisms to induce tolerization.

CD28 is the most prominent co-stimulatory molecule for T-cell activation and differentiation. It controls the activation of both, naive and memory T-cells. Binding of CD86 to CD28 is regarded as the main T cell co-stimulatory interaction [10]. Blocking the CD28/86 pathway has been used as a strategy for inducing tolerance in chronic autoimmune diseases [9]. We showed that surface expression of CD28 on blebs derived from activated lymphocytes is not significantly increased by PUVA treatment. Rather, starting as early as 12 hours after treatment, blebs with an even decreased expression of CD28 are released from the dying cells. The same was seen for CD86, at even earlier time points after treatment. Taken together, blebs released after PUVA treatment display an immunosuppressive phenotype, characterised by an increased exposure of CD5 and a decreased exposure of CD28 and CD86. Holtick and colleagues showed that PUVA-treated DC skewed naive T cells toward a more anti-inflammatory Th2 response [12]. Our data further confirm the hypothesis that modulation of cell death contributes to the immune regulatory effects of ECP and that blebs are also involved in this process. Alternative treatments in steroid-resistant cGVHD are urgently needed [5].

We conclude that immune suppressive apoptotic cells and during cell death released blebs, both induced by ECP, should be considered as one additional treatment option in inflammatory diseases.

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7 Abkürzungsverzeichnis

(c)GVHD	(chronic) graft-versus-host-disease
*	$p < 0.05$
**	$p < 0.01$
8-MOP	8-methoxypsoralen
AxV	annexin V
cts.	counts
DC	dendritic cells
ECP	extracorporeal photopheresis
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
IL10	interleukin-10
IL2	interleukin-2
MnX	mean value
neg.	negative
PBMC	human peripheral blood mononuclear cells
PE	phycoerythrin
PHA	phytohemagglutinine
PI	propidium iodide
pos.	positive
PS	phosphatidylserine
PUVA	treatment with Psoralen plus UV-A-light
SD	standard deviation
w/o	without

8 Anhang

Figure 1

Apoptosis and necrosis of stimulated lymphocytes after treatment with 8-MOP and/or UV-A.

[A-C] The exposure of PS by activated lymphocytes 12h after treatment with 8-MOP, UV-A, or PUVA was analysed by staining with AxV-FITC, and the membrane permeability by counter staining with PI. The respective cell cycle and the nuclear degradation (sub G1) are displayed in [D-F]. *Note:* PUVA treated lymphocytes [C, F] showed a much higher rate of apoptosis (AxV-FITC positive/PI negative cells [C] and sub G1 content [F]) as well as of necrosis (AxV-FITC positive/PI positive cells [C]) than the 8-MOP only [A, D] or UV-A [B, E] treated cells. Primary data of one representative experiment

out of five is displayed. Time kinetics of apoptosis [G] and necrosis [H] of activated human lymphocytes was also monitored by flow cytometry. Data of one representative set of experiment out of five, each performed in triplicates, are displayed; ** $p < 0.01$. AxV: annexinV, neg.: negative, PHA: phytohemagglutinine, PI: propidium iodide, pos.: positive, PS: phosphatidylserine, 8-MOP: 8-methoxypsoralen, PUVA: 8-MOP plus UV-A.

Figure 2

Time course of apoptosis of lymphocytes after treatment with 8-MOP and/or UV-A.

[A] Apoptosis was monitored by staining with AxV-FITC and PI and [B] by detecting the nuclear degradation (sub G1). The time course of apoptosis of activated (continuous line) and non-activated (dashed line) human lymphocytes was monitored by flow cytometry. *Note:* PUVA treated activated as well as non-activated lymphocytes show a significant higher amount of apoptosis than UV-A or 8-MOP treated cells. Data of one representative set of experiment out of five are displayed. AxV: annexinV, PHA: phytohemagglutinine, PI: propidium iodide, 8-MOP: 8-methoxypsoralen, PUVA: 8-MOP plus UV-A, w/o: non-stimulated.

Figure 3

Time course of the release of blebs of lymphocytes after treatment with 8-MOP and/or UV-A.

Stimulated lymphocytes [B] show a higher release of blebs in comparison to non-stimulated ones [A]. In all cases, PUVA treatment led to a significant enhanced number of blebs. Even 8-MOP treatment only could increase the number of sub-cellular fragments. Data of one representative set of experiment out of five, each performed in triplicates, are displayed; * $p < 0.05$; ** $p < 0.01$. cts: counts, 8-MOP: 8-methoxypsoralen, PUVA: 8-MOP plus UV-A.

Figure 4

Phosphatidylserine exposure and surface antigen composition of blebs of lymphocytes after treatment with 8-MOP and/or UV-A.

[A] PS exposure was determined by AxV-FITC binding on blebs and detected by flow cytometry. *Note:* AxV positive blebs are mainly derived from stimulated cells. Data of one representative set of experiment out of five, each performed in triplicates, are displayed. [B-E] The surface antigen composition of the blebs was monitored by flow cytometry. *Note:* All CD5 [B] and CD8 [C] values differed significantly at all time points between the different treatments in the case of stimulated cells. 24 hours after treatment the expression of CD28 [D] on blebs of non-stimulated cells was higher than on stimulated ones and that of CD86 [E] was higher on blebs of non-stimulated lymphocytes at each time of measurement. Data of one representative set of experiment out of five, each performed in triplicates, are displayed. AxV: annexinV, cts: counts, MnX: mean value, PHA: phytohemagglutinine, pos.: positive, 8-MOP: 8-methoxypsoralen, PUVA: 8-MOP plus UV-A, PHA: phytohemagglutinine, w/o: non-stimulated

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