Pathogen inactivation in cellular blood products by photodynamic treatment

Trannoy, L.L.

CHAPTER VII

IMPACT OF PHOTODYNAMIC TREATMENT WITH MESO-SUBSTITUTED PORPHYRIN ON THE IMMUNOMODULATORY CAPACITY OF WHITE BLOOD CELL-CONTAINING RED BLOOD CELL PRODUCTS

Laurence L. Trannoy[*1], Dave L. Roelen[2], Karin M. Koekkoek[2], Anneke Brand[1,2]

[1]Department of Research and Education, Sanquin Blood Bank South-west, Leiden, the Netherlands; [2]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands

Accepted for publication in Photochemistry and Photobiology

ABSTRACT

After transfusion, the presence of contaminating white blood cells (WBC) in blood components may result in either deleterious or positive immunological responses. We have previously reported that PDT with meso-substituted mono-phenyl-tri-(N-methyl-4-pyridyl)-porphyrin (Tri-P(4)) and red light can inactivate pathogens in RBC products. The present study explored the effect of PDT on contaminating WBC in RBC products with varying hematocrit (Hct). After PDT, we evaluated adaptive and innate immunomodulation through allogeneic and mitogenic stimulation. PDT resulted in decreased T cell proliferation which was more pronounced with lower Hct. Dark effect of porphyrin Tri-P(4) was remarkable on antigen-presenting cells affecting expression of co-stimulatory molecules CD80/CD86. Finally, cytokine profile after PDT revealed a mixed Th1/Th2 type response while surface antigen expression supported the development of alternatively activated macrophages (AAMϕ or type 2 macrophages) instead of dendritic cells. In conclusion, PDT with Tri-P(4) altered proliferation, allo-stimulation, cell surface antigen expression and cytokine profiles of the cells. These results suggest that PDT may be potentially useful in preventing transfusion associated Graft-versus-Host Disease and alloimmunisation. It seems worthwhile to further explore PDT-induced immunomodulation to optimize conditions which may result in allo-tolerance by AAMϕ.

INTRODUCTION

Risks associated with blood transfusion has dramatically decreased since the implementation of careful donor selection and the development of sensitive diagnostic tests, however a certain risk for complications by contaminating pathogens still remains.[1,2]

Not only are viruses, bacteria and protozoa considered as pathogenic, allogeneic WBC present in blood components can also be recognized as pathogenic contaminants, inducing immune responses in the recipient, sometimes with deleterious effects. Such WBC-induced complications of blood transfusion include transfusion associated Graft-versus Host disease (GvHD), transfusion related immunosuppression, which can result in enhanced susceptibility to post-operative infection and multiple-organ-dysfunction syndrome, non-hemolytic febrile transfusion reactions (NHFTR) and immunization to Human leukocyte antigens (HLA).[3] Although the mechanism is yet to be unraveled, the presence of allogeneic WBC in transfusion products can be beneficial reducing the risk of allograft rejection and of spontaneous recurrent abortion.[3]

A number of approaches have been implemented to minimize the pathogenic role of contaminating WBC. Leukodepletion of blood components by filtration has resulted in a reduction in the frequency of NHFTR's and HLA immunisation.[2] Gamma irradiation is effective in preventing WBC proliferation but does not impair the stimulatory capabilities of these cells.[4] Some of these techniques, aimed to inactivate pathogen in blood products, such as Mirasol-Pathogen-Reduction technology[5] and Inactine technology using Pen 110[6] have been shown to also reduce the proliferative capacity of the WBC as well as their ability to present antigens. Furthermore, it has been shown that photodynamic treatment (PDT) can have immunomodulatory effects in various experimental systems. In-vivo studies showed that PDT with verteporfin was effective in alleviating immune pathology in murine models of arthritis, contact hypersensitivity, experimental allergic encephalomyelitis and retention of allogeneic skin grafts.[7,8] PDT of peripheral blood mononuclear cells with a rhodamine derivative TH9402 may induce tolerogenic dendritic cells (DC)[9], whereas treatment with extracorporeal

therapy using 8-methoxypsoralen was found to reverse GvHD.[10] Investigations in the mechanism involved in immunomodulatory PDT have shown that PDT selectively eliminate certain cell populations, disrupt T cell response by affecting APCs by lowering expression of key cell surface molecules, including MHC I and II, CD80, CD86 and CD54 and modulate the cytokine network.[11-13]

PDT utilizes mainly porphyrin and phthalocyanine-based photosensitizers which accumulates somewhat selectively in rapidly dividing cells such as activated cells of the immune system.[14-16] Exposure to sub-lethal doses of PDT may result in the modification of cell surface receptor expression levels and cytokine release and consequently influence cell behaviour.[8]

Mono-phenyl-tri-(N-methyl-4-pyridyl)-porphyrin chloride (Tri-P(4)) is a positively charged amphiphilic porphyrin that can be activated with red light to generate singlet oxygen responsible for oxidative damage to vicinal molecules. We have previously demonstrated that this photosensitizer was superior to other similar porphyrin photosensitizers in its action to efficiently inactivate a wide range of viruses and bacteria with limited damage to RBC.[17,18] PDT with Tri-P(4) was also shown to have limited effects on cord blood derived stem cells and progenitor cells.[19] These observations raised the question as to whether this treatment may be able to modulate WBC present in RBC product to reduce immunological post-transfusion reactions or to enhance beneficial tolerizing effects of blood transfusion.

We therefore explored the potential immunological effects of PDT with Tri-P(4) on contaminating WBC in RBC products with varying Hct. For this, we evaluated the effect of the treatment on in-vitro adaptive and innate immunological parameters, such as proliferation in response to mitogens and to allogeneic stimulator cells, the ability to stimulate proliferation of healthy untreated responder cells, survival, expression of constitutive and inducible cell surface antigens and production of soluble mediators (cytokines).

MATERIALS AND METHODS

MATERIALS

Photosensitizer Tri-P(4) (mono-phenyl-tri-(N-methyl-4-pyridyl)-porphyrin chloride) was kindly provided by Buchem b.v., Apeldoorn, the Netherlands. Stock solutions of 1 m*M* were prepared in PBS and stored in the dark at 2-6°C. PBS (pH 7.4) and Ficoll gradient were provided by the Leiden University Medical Center (LUMC) Pharmacy, Leiden, the Netherlands. SAG-M is a red cell storage solution containing 150 m*M* NaCl, 1.25 m*M* adenine, 50 m*M* glucose and 29 m*M* mannitol (Fresenius Hemocare, Emmer-Compascuum, the Netherlands). IMDM and RPMI-glutamine was purchased from Invitrogen, Breda, the Netherlands. Penicillin and streptomycin (pen/strep) were purchased from Bio-Whittaker (Verviers, Belgium). Pools of 40 individual AB sera, screened for the absence of HLA antibodies and disturbance in mixed lymphocyte reaction (MLR), were obtained from Sanquin Blood Bank Southwest (Rotterdam, the Netherlands). Phytohemagluttin (PHA), lipopolysaccharide (LPS) and β-mercapto-ethanol were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Phycoerythrin (PE)-conjugated anti-human CD14, CD16, CD33, CD54, CD80, CD83, CD86, HLA-DR monoclonal antibodies (MoAb), PE-conjugated isotype-matched mouse IgG2a MoAb, fluorescein (FITC)-conjugated anti-human CD45 MoAb, FITC-conjugated isotype-matched mouse IgG1 MoAb, propidium iodine (PI), IOTest3 lysis solution and the Flow-count fluorospheres were purchased from Beckman Coulter (Mijdrecht, the Netherlands).

BLOOD PRODUCTS AND PROCESSING

Whole blood derived buffy coats were provided by healthy HLA-typed donors from Sanquin Blood bank supply (Leiden, the Netherlands). The buffy coats were washed twice at 5000 gmin with SAG-M to remove plasma. After the last wash, the supernatant and the WBC layer were transferred into a new tube and centrifuged at 8500 gmin. The WBC pellet was resuspended in 10 mL SAG-M for counting. Subsequently, the WBC and SAG-M were added to the washed RBC to

obtain a final product with a WBC concentration 20.10^6 cell / mL and a Hct of 20% ± 4% or 55% ±5%. Hct and cell concentrations were measured by Act 10 blood analyzer (Beckman Coulter, Mijdrecht, the Netherlands).
The final product obtained is referred to as WBC-enriched RBC (WBC-RBC). All samples were DNA typed at low resolution for the loci HLA-A, -B, -C, -DRB1 and -DQB1 by polymerase chain reaction /sequence-specific oligonucleotide using a reverse dot-blot method.[20] HLA typing was performed at the national reference laboratory for histocompatibility testing (LUMC, the Netherlands).

LIGHT SOURCE

For all illuminations, the light source was a 300 W halogen lamp (Philips, Eindhoven, the Netherlands) combined with a cut-off filter (Kodak, wratten nr. 23A, Rochester, NY) only transmitting light > 600 nm. The fluence rate at the level of the Petri dish was adjusted to 100 W per m^2, as measured with an IL1400A radiometer with a SEL033 detector (International Light, Newburyport, MA). To avoid heating of the samples, the light was passed through a 1-cm thick water layer. The temperature did never exceed 25°C.

PHOTODYNAMIC TREATMENT

Tri-P(4) was added to the blood components to a final concentration of 25 µ*M*. The WBC-RBC suspensions were thoroughly mixed. Subsequently 3 mL samples were transferred into 6 cm diameter petri dishes (tissue culture grade, Greiner, Alphen a/d Rhijn, the Netherlands), resulting in a cell layer thickness of 1 mm. The dishes were agitated at room temperature on a horizontal circular shaker (75 rpm) for 5 min in the dark and subsequently illuminated under continuous agitation for 30 min corresponding to a light of 180 kJ/m^2. The light control corresponded to WBC-RBC illuminated without addition of photosensitizer. The dark control corresponded to WBC-RBC spiked with photosensitizer but kept in the dark during the whole procedure (including preincubation and illumination time).

MITOGENIC STIMULATION

To study proliferation, mononuclear cells (MNC) were isolated from WBC-RBC immediately after PDT by centrifugation on Ficoll density gradient and subsequently, the cells were resuspended in culture medium containing RMPI-Glutamine supplemented with 10% human serum and 100 µg/mL Pen/strep. The cells (10^6 cell/mL) were stimulated with 1 µg/mL PHA in U-bottom 96-well plates. Cell proliferation was quantified by incubating the cells during the last 18 h of 4-day cultures with 1 µCi [^{3}H]thymidine. Before being pulsed, supernatants were collected and stored at –40°C. Proliferation was measured as [^{3}H]thymidine incorporation by liquid scintillation spectroscopy using a beta plate (Wallac, Turku, Finland). Results are expressed as % of light control.

MIXED LYMPHOCYTE REACTION

To study allo-reactivity, MNC isolated from WBC-RBC after PDT by Ficoll isolation, as described above, were co-cultured with MNC isolated from a completely HLA-mismatched buffy coat (allogeneic cells). Equal numbers of responder and stimulator cells (100 µL of 10^6 cells/mL) were cultured in U-bottom 96-well plates (Costar, Cambridge, MA, USA). Stimulator cells were obtained by irradiation at 3000 Rad. Tri-P(4)-treated cells, controls and allogeneic cells were used both as responders and as stimulators. As a control for spontaneous proliferation, the responder and stimulator cells were cultured in medium only. Cell proliferation was quantified by incubating the cells during the last 18 h of 5-day cultures with 1 µCi [^{3}H]thymidine (Amersham International, Amersham, UK). Assays were performed in triplicate. Before being pulsed, supernatants were collected and stored at –40°C. Proliferation was measured as described above. Results are expressed as % of light control.

LPS STIMULATION

After PDT, WBC-RBC was washed 4 times at 5000 gmin to remove the photosensitizer and, subsequently diluted 10-fold with IMDM supplemented with 100 µg/mL Pen/strep and 50µ*M* beta-mercapto-ethanol. For stimulation with LPS,

cell suspension (150 µl/well) were plated in triplicate in plate-bottom plate (Costar, Cambridge, MA, USA) and 50 µl of LPS (final concentration 250 pg/mL) or culture medium was added. After 20 h of incubation at 37°C, 5% CO_2, samples were pooled then centrifuged at 2700 gmin. Supernatant was harvested and frozen at -40°C until use. Supernatant from non-LPS treated cell suspensions were harvested and frozen to be used as reference values before stimulation.

CYTOKINE ANALYSIS

Harvested supernatants from MLR and LPS stimulation assays were tested for a total of 17 cytokines at once (Interleukin-1beta (IL-1 β), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-17, Granulocyte-colony stimulating factor (G-CSF), Granulocyte-monocyte-CSF (GM-CSF), Interferon-gamma (IFN-γ), monocytes chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β) and tumor necrosis factor-alpha (TNF-α) with the Bio-Plex human Cytokine panel following the manufacturer's description. Samples were analyzed using a Bio-Plex array reader equipped with Bio-Plex software (Bio-Rad Laboratories, Veenendaal, the Netherlands).

IMMUNOPHENOTYPING

After PDT application of WBC-RBC, samples were washed 4 times with PBS at 1500 gmin to remove the photosensitizer. Cells were incubated with anti-human MoAb for 20 min at room temperature. After red cell lysis with lysis solution IOTest3, samples were analyzed on a four-laser cytometer EpicsXL-MCL flow cytometer using Expo32 software (Beckman Coulter, Mijndrecht, the Netherlands).

CELL VIABILITY

Using PI labeling and flow cytometry, cell viability was assayed in cell cultures from MLR at day 1, 4 and 6; day 0 being the day of input. Unstained cells and cells labeled with PE-conjugated isotype-matched mouse IgG2a and FITC-conjugated IgG1 of irrelevant specificity were used as negative controls.

STATISTICS

For statistical analysis, Repeated measured Analysis of Variance (ANOVA) or Friedman test were used when appropriate, depending on the type of distribution. P values are from comparison with light controls and determined by Tukey's or Newman-Keuls post-tests as indicated in legends. P values < 0.05 were considered as statistically significant. All data are expressed as Mean ± Standard Deviation (sd).

RESULTS

EFFECT OF PDT ON PROLIFERATION

After PDT application, MNC were tested for their capacity to proliferate in response to PHA (Fig.1A), and to allogeneic stimulation (Fig.1B). PDT induced a significant reduction in proliferation when samples were treated in low Hct level. After stimulation with PHA, no significant difference was observed between light and dark controls. However, after PDT at low Hct level and allogeneic stimulation, we observed a significant increase in proliferation in dark controls as compared to light controls. The spontaneous proliferation of the photo-treated cells and dark control cells did not differ from the light controls (not shown).

EFFECT OF PDT ON THE ABILITY OF MNC TO PRESENT ANTIGEN

After PDT application, MNC were tested for their capacity to present antigen and thereby stimulate a
proliferative response in untreated HLA-mismatched allogeneic cells. As can be seen from Fig.1C, the proliferative response induced by photodynamically treated stimulator cells was significantly reduced by approximately 50% of the light controls when the cells were treated in low Hct level. In high Hct level, no difference between the samples was seen. In the dark samples, no difference in the proliferative response was seen as compared to light controls. The spontaneous proliferation of the irradiated stimulator cells did not differ between the samples (not shown).

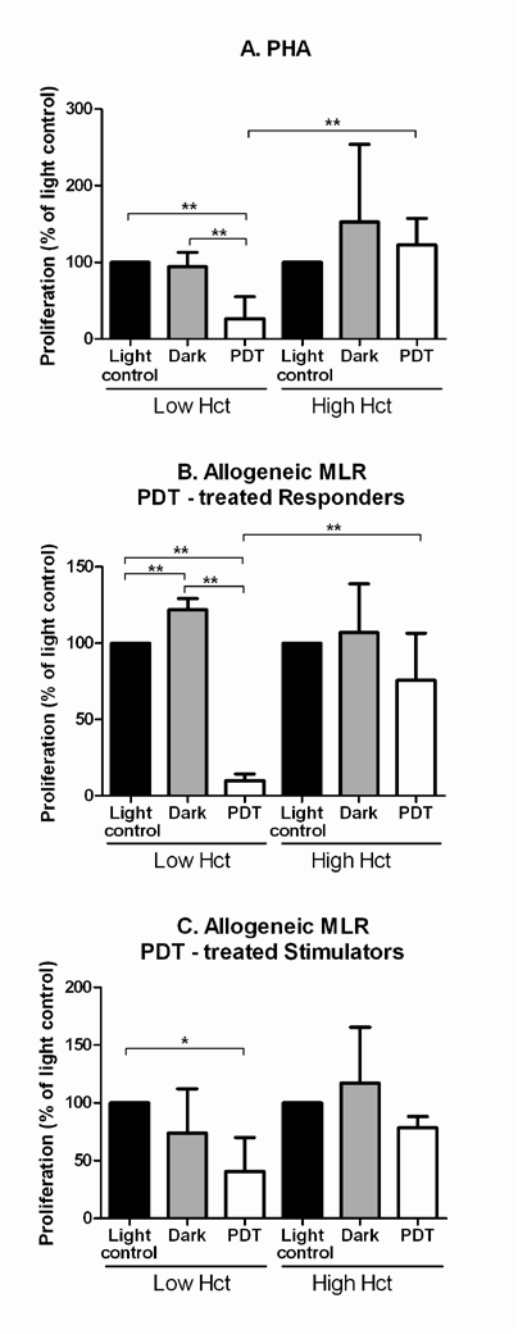


Figure 1. Proliferation capacity of MNC after PHA stimulation (A), and allogeneic stimulation (B) after PDT of WBC-RBC in low (~ 20%) and high (~ 50%). (C) Capacity of photo-treated MNC to stimulate proliferation in MLR.

Proliferation is expressed as % of light control. Mean with standard deviation are shown. Light controls (black bars), Dark samples (gray bars), PDT samples (white bars). Statistic differences between the three groups of samples within each Hct level were performed using ANOVA and Tukey-post tests. Comparisons of each group between the two Hct levels were performed using student's *t*-test. (*) p values < 0.05; (**) p values < 0.01.

THE EFFECT OF PDT ON CELL VIABILITY

Immediately after PDT of WBC-RBC, MNC were isolated by Ficoll centrifugation. Cell viability was determined during MLR at day 1, day 4 and day 6 of culture to check that the proliferation inhibition induced by PDT may not be due to excessive apoptosis induction. PDT did not affect the overall cell viability. The % of PI-positive cells was 10-15% at day 1 and increased to 25-30% at day 6 of the MLR culture and was similar to untreated controls. There was no difference in non-viable cells in cultures where photodynamically treated cells were used as responders or in cultures where the treated cells were used as stimulator cells. In addition, no difference was seen between light controls and dark controls (data not shown).

EFFECT OF PDT ON THE RELEASE OF CYTOKINES BY ALLO-ACTIVATED PHOTO-TREATED RESPONDER CELLS IN MLR

The cytokine pattern produced in MLR by photodynamically treated MNC used as responders was determined. As the effect of PDT on the proliferation was dependent on the Hct level of the treated product, a similar relationship was observed with the cytokines released during the cultures. When MNC were treated in low Hct level (<30), a significant negative correlation was observed between proliferation and the release of IL-4, IL-10, IL-13, IL-12, TNF-α, MIP-1β and INF-γ (Fig.2); the lower the proliferation, the more cytokines were released. In contrast, the release of IL-2 was positively correlated with proliferation; the lower the proliferation, the lower IL-2 was released. In high Hct level (>50), no significant change in cytokine release in PDT samples was seen as compared with light controls. Moreover, for IL-1β, IL-5, IL-6, IL-7, IL-8, IL-17, G-CSF, GM-CSF, MCP-1, no significant difference with light controls was observed at both Hct levels (data not shown). Finally, no difference in cytokine pattern between dark and light controls was seen (data not shown).

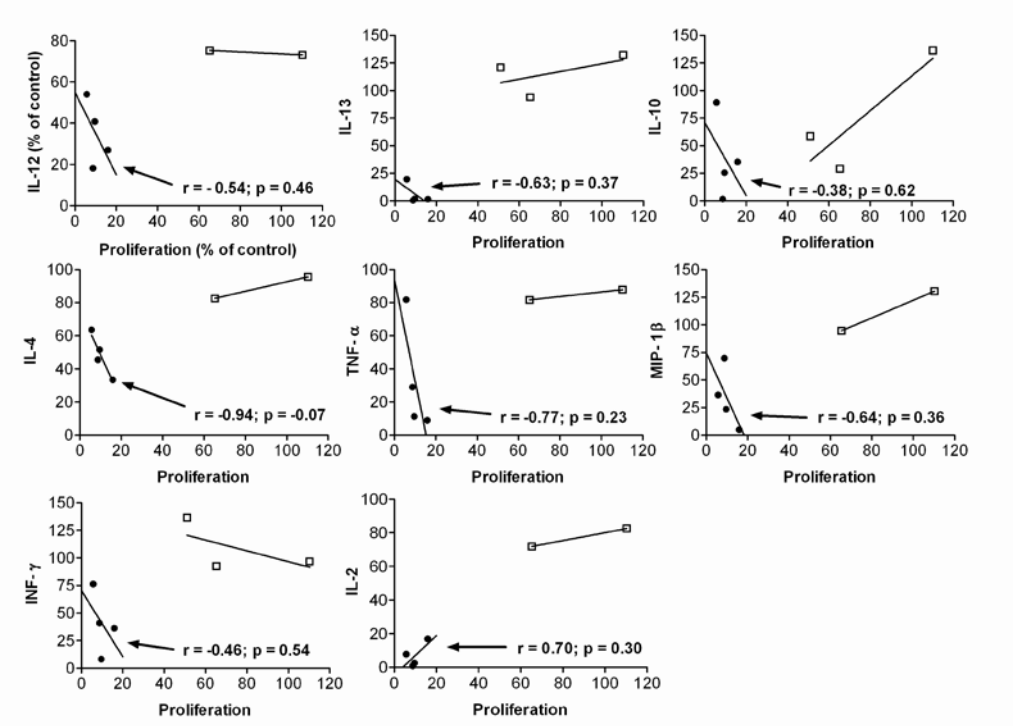


Figure 2. Correlation between proliferation and cytokine release in MLR wherein MNC were used as responders after PDT of WBC-RBC in Hct <30 (circles) or in Hct > 50 (squares). Both level of released cytokine and proliferation rate are expressed as % of light control. Only PDT samples are represented. Results shown are mean of triplicates of 6 independent experiments, each one represented by one dot. Correlations were indicated by Pearson r and p value. Fit lines were established by linear regression.

EFFECT OF PDT ON THE RELEASE OF CYTOKINES BY TRI-P(4)-TREATED STIMULATOR CELLS IN MLR

The cytokine pattern produced in MLR by allogeneic cells after antigen presentation by Tri-P(4)-treated MNC was determined. In concordance with the proliferation results, the level of cytokine released did not correlate with the Hct level of the product during PDT. However, as can be seen from Fig.3, significant negative correlations between proliferation and the release of IL-1β, IL-6, INF-γ, TNF-α, MIP-1β and GM-CSF were observed. Striking was that the cytokine levels mostly exceeded the levels of light controls (> 100%) when proliferation was the

most inhibited, except for IL-2 showing a significant positive correlation with proliferation. In addition, no difference in cytokine pattern between dark and light controls was seen (data not shown).

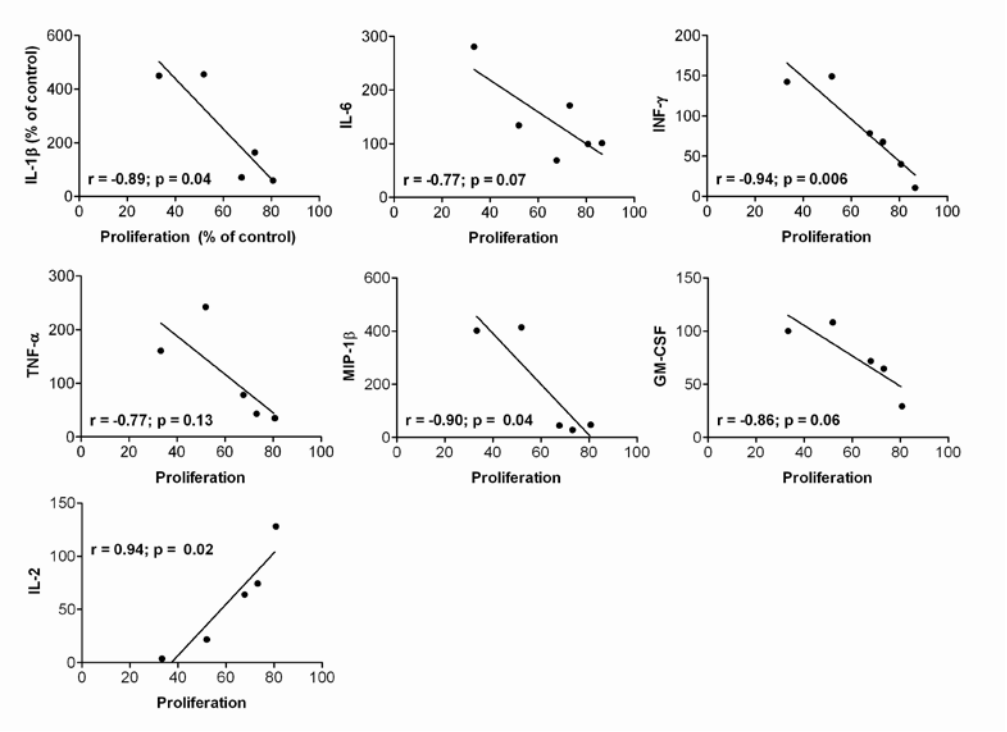
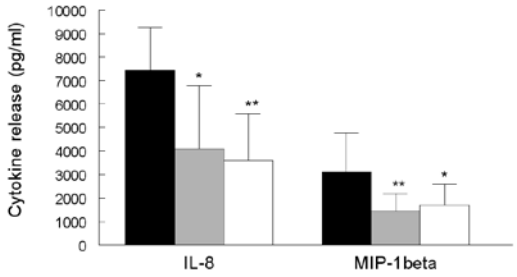


Figure 3. Correlation between proliferation and cytokine release in MLR wherein MNC were used as stimulators after PDT of WBC-RBC.
Both level of released cytokine and proliferation rate are expressed as % of light control. Only PDT samples are represented. Results shown are mean of triplicates of 5 or 6 independent experiments, each one represented by one dot. Correlations were indicated by Pearson r and p value. Fit lines were established by linear regression.

EFFECT OF PDT ON LPS-STIMULATED CELLS

After PDT application, WBC-RBCs were stimulated with LPS for 20 hours, after which cytokine production and antigen expression were determined. As can be seen in Fig.4, in dark and PDT samples, a significant reduction of nearly 50% in release of IL-8 and MIP-1β as compared to light controls was observed (graph Fig.4) whereas for the other cytokines tested no difference with the light controls was observed (frame in Fig.4).
The proportion of cells expressing surface antigen were determined and given in Fig.5. PDT induced an increase in numbers of CD14, HLA-DR and CD83 expressing cells as compared to light and dark controls. The increased in the amount of HLA-DR and CD83 positive cells was only observed after LPS stimulation, while the increased in the amount of CD14 positive cells was LPS independent. Moreover, four hours after PDT application and before LPS stimulation, the amount of CD80 and CD86 expressing cells decreased significantly as compared to light controls as well in the dark samples as in PDT samples. No PDT effect and LPS effect was observed in the amounts of CD33, CD54 and CD16 expressing cells (Fig.5).
Moreover, alteration in the stimulation capabilities of APC was associated with increased production of cytokines suggestive of active inhibition. Investigation of the innate immunity of WBC in treated RBC products using LPS showed that Tri-P(4) alone affect the APC response.

Changes in cellular expression of cell surface markers were determined by measuring mean fluorescence intensities (MFI) of each marker and given in Table 1. The MFI of CD14 and HLA-DR expressions increased upon PDT as compared to light and dark controls and both were LPS independent. In contrast, MFI of CD83 expression increased upon PDT only without LPS, while after LPS stimulation, MFI remained similar between the three groups of samples. No significant change in MFI of CD33, CD16, CD54, CD80 and CD86 expressions were observed after PDT as compared to light and dark controls.



	Light control	Dark	PDT
IL-1β	568 ± 699	566 ± 644	568 ± 642
IL-2	23 ± 18	23 ± 14	20 ± 10
IL-4	56 ± 47	45 ± 29	41 ± 23
IL-6	6578 ± 3734	6780 ± 5186	7123 ± 2896
IL-7	3.1 ± 1.9	2.8 ± 1.8	2.6 ± 0.9
IL-10	119 ± 102	137 ± 162	133 ± 119
IL-12	3.1 ± 2.5	4.2 ± 3.7	3.0 ± 1.6
IL-17	27 ± 9.0	25 ± 9.4	23 ± 6.2
INF-g	150 ± 116	175 ± 113	161 ± 88
TNF-a	203 ± 217	214 ± 204	210 ± 127
MCP-1	856 ± 984	716 ± 538	1190 ± 906
G-CSF	1004 ± 922	687 ± 474	778 ± 446
GM-CSF	145 ± 142	134 ± 94	118 ± 67

Figure 4. Effect of PDT on the release of cytokines in LPS-stimulated cells. Cytokine releases are expressed in pg/mL. Mean ± sd of 6 independent experiments are shown. Asterisks indicates statistical differences with the light controls as determined by ANOVA and Tukey post-test (*) p values < 0.05; (**) p values < 0.01. Graph legend: Light controls (black bars), dark controls (gray bars); PDT (white bars).

DISCUSSION

PDT with Tri-P(4) and red light has been shown to inactivate a number of pathogens including viruses and bacteria in RBCC[18] and cord blood products.[19] As contaminating WBC in RBCC are implicated as a causative factor for an immune response after transfusion[3,21], we were interested to determine the effect of Tri-P(4)-mediated PDT on these cells.

PDT with Tri-P(4) had no effect on the spontaneous proliferation and cell viability of the treated WBCs. However, photodynamically treated WBCs showed impaired proliferation after stimulation with PHA and upon co-culture with allogeneic stimulator cells. The degree of impairment of proliferative capacity was increasing when WBC had been exposed to PDT in lower Hct. Although cytokine production was reduced at a certain level of proliferation inhibition, stronger proliferation inhibition resulted in less decrease of cytokine production (Fig.2). PDT also impaired the capacity of antigen-presenting cells (APC) to stimulate the T-cell proliferation in MLR, however, this effect was less influenced by Hct. Moreover, the reduction in the stimulation capabilities of APC was associated with increased production of cytokines suggestive of active inhibition. We also found by using LPS that the APC response was affected by the presence of Tri-P(4) only, in the absence of light.

	Medium			LPS		
	Light control	Dark	PDT	Light control	Dark	PDT
HLA-DR	107 ± 89	114 ± 63	210 ± 139*	136 ± 129	99 ± 73	208 ± 157*
CD83	30 ± 12	33 ± 7.6	41 ± 6.7*	26 ± 12	26 ± 11	28 ± 8.7
CD14	729 ± 234	886 ± 271	1016 ± 404†	773 ± 187	709 ± 249	1174 ± 411†
CD33	39 ± 12	39 ± 17	35 ± 16	52 ± 24	49 ± 29	48 ± 20
CD16	32 ± 12	31 ± 7.4	31 ± 6.6	32 ± 8.4	31 ± 12	40 ± 23
CD54	87 ± 95	106 ± 130	76 ± 57	215 ± 272	147 ± 178	166 ± 160
CD80	62 ± 98	42 ± 29	45 ± 19	34 ± 3.7	27 ± 6.7	41 ± 7.4
CD86	69 ± 29	97 ± 73	110 ± 55	59 ± 48	52 ± 27	66 ± 24

Table 1: Intensities of cell surface marker expression (MFI) on WBC after PDT
Data indicated are Mean Fluorescence Intensity (MFI) ± SD of 8 independent experiments. Statistic differences were determined with ANOVA and Newman-Keuls post tests or with Friedman test depending on the type of distribution. Tests were performed in respective medium. Superscript symbols shown indicate a significant difference with light controls ($p < 0.05$): (*) from ANOVA and ($\dagger$) from Friedman test.

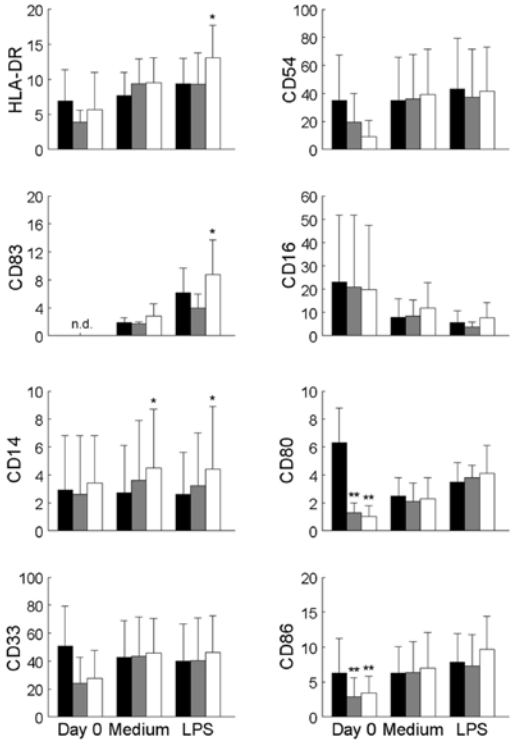


Figure 5. Effect of PDT on the cell surface expression on LPS-stimulated WBC-RBC.
Cell surface antigen expression is expressed as percentage of positive cells from the CD45+ population. Day 0 corresponds to un-stimulated WBC-RBC 4 hours after PDT. Medium and LPS corresponded to WBC-RBC cultured after PDT without and with LPS respectively for 20 hours.
Light controls (black bars), Dark controls (gray bars), PDT (white bars). Mean ± sd of 8 independent experiments are shown. Asterisks indicates statistical differences with the light controls in their respective culture media as determined by ANOVA and Tukey post-test; (*) p values < 0.05; (**) p values < 0.01; n.d. not detectable.

The dark effect of the photosensitizer Tri-P(4) on APCs was compatible with our observations that Tri-P(4) binds non covalently but differentially to leukocytes with a preference for monocytes/macrophages and granulocytes (L.L. Trannoy, unpublished). We observed that release of both chemoattractants IL-8 and MIP-1β were reduced as well in dark and in PDT samples. It has been reported that,

the release of these cytokines have in common a signaling pathway.[22,23] Further studies are required to identify the membrane receptor which may interact with Tri-P(4) disrupting simultaneously the release of IL-8 and MIP-1 β. Moreover, the expression of co-stimulatory molecules CD80 and CD86 on APC were also reduced both in dark and PDT samples (Fig.5). These results suggest that Tri-P(4) affects the antigen presentation functionality of APC by interacting directly with monocytes / macrophages in the absence of light.

The Hct of the blood product influences the photodynamic effect on T cells. Increasing the Hct caused higher pigmentation of the blood product and may hinder proper light penetration to the WBC. Thereby PDT may affect differently the WBCs. High doses and low doses PDT has been shown to generate different results as high doses resulted in necrotic or apoptotic cells, while low doses PDT or sublethal doses PDT may result in the modification of cell surface receptor expression and consequently influence cell activities.[8,24]

Another possible explanation is the capacity of RBC to scavenge reactive oxygen species (ROS).[25] Tri-P(4) is a porphyrin that remains extracellular by binding exclusively to cell membrane of living and resting cells (unpublished results, L.L. Trannoy). Illumination of porphyrin Tri-P(4) at appropriate wavelength generates ROS, in particularly singlet oxygen ($^1O_2$) which reacts with substances in close vicinity. ROS is also a natural product, produced by neutrophils in host defense to kill invading pathogens and involved in intercellular and intracellular signaling of homeostasis, cell proliferation and differentiation and inflammatory and immune responses.[26] Generation of ROS by light activation of Tri-P(4) can cause lipid and amino-acid peroxidation, affecting cell membrane associated targets, and may produce a number of effects on the immune cells. RBC may play a protective role by scavenging PDT-generated extracellular ROS and varying the Hct of RBC may affect the degree of photo-damage induced by ROS. Further research would determine whether illumination with a lower light dose in the absence of RBC may show the same effects on WBC after PDT with Tri-P(4).

In our study, T-cell response and IL-2 release correlated with the Hct level of the photodynamically treated WBC-RBC products, as seen after PDT when Tri-P(4)-

treated cells were used as responders in MLR and after PHA stimulation. This indicated that T-cells are target for ROS-mediated PDT and that the presence of RBC during PDT application may protect the T cells against the damaging effect of singlet oxygen. Possible targets of PDT may be the TCR and CD28 signaling pathways which were found to be sensitive to oxidative stress induced e.g. by BSO (DL-buthionine-(S,R)-sulfoximine) depleting intracellular antioxidant reduced glutathione [27] and after PUVA treatment.[28] Both treatments result in T-cell hyporesponsiveness.

Our results showed that PDT with Tri-P(4) act both on APCs and on T cells, on one hand through a dark effect of Tri-P(4) down-regulating the costimulatory molecules CD80 and CD86 and on the other hand through a photodynamic effect inhibiting the responder and stimulatory potentials of WBC in MLR. Therefore we suggest that the immunomodulatory effect of PDT may result in an impaired interaction between T-cells and APC. Blockage of the CD80/CD86-CD28 co-stimulation was shown to result in T cell hyporesponsiveness and in generation of alternatively activated macrophages (AAMϕ), also referred to as type 2 macrophages [29-31] The induction of this type of macrophages is supported on one hand by the PDT-induced increase in cell number and cellular expression of HLA-DR and CD14 on APC, and on the other hand by the refractoriness of CD80 / CD86 up-regulation to LPS stimulation (Table 1). These cellular events are partly characteristic of AAMϕ as demonstrated by Xu [32] and Verreck.[30] As these cells are unable to prime T-cells, they may contribute to the PDT-induced T-cell hyporesponsiveness.[31,33] An additional indication pointing toward the involvement of APC after PDT is the fact that un-stimulated WBC-RBC cultures of photo-treated cells showed an up-regulation of CD83 which was refractory to LPS stimulation (Table 1). Indeed, monocytes are precursors of dendritic cells (DC) and macrophages, with each cell type having the capability to convert into the other until late in differentiation / maturation process.[34] Hence, PDT seems to activate monocytes to differentiate into DC as suggested by the high expression of CD83 and HLA-DR (Table 1); however, LPS stimulation skews this polarization favoring type 2 macrophage development (lower CD83, higher CD14 expression).[30]

Finally, at a level of PDT-induced proliferation reduction up to 40%, it appeared that the allo-stimulated cells released increasing cytokine levels proportionally to

the degree of inhibition of proliferation; cytokine levels exceeding even these in light control cultures (Fig.3). These results strongly suggest an immunomodulatory event. Moreover, the cytokine profile revealing a mixed Th1/Th2 type response, suggests that several types of APC may be synergically or simultaneously involved.

In conclusion, in addition to inactivating pathogens, PDT with Tri-P(4) may be able to steer the immune response to T-cell hyporesponsiveness by functionally affecting both T cells and APC. These dual effects may present an advantage on gamma irradiation which was shown to inhibit proliferation of the MNC without affecting the antigen-presenting capabilities of the irradiated cells.[35] Consequently, we may consider the possibility of using PDT with Tri-P(4) to deviate an immune response towards tolerance or to prevent undesired transfusion related immunological reactions.

Acknowledgments--This work was supported by ZonNW (grant nr. 28-24140), the Netherlands. The authors thank Aine Honohan for her technical supports and her helpful English editing. The authors thank also Johan Lagerberg for the critical review of this article.

REFERENCES

1. Barbara J. Why 'Safer than ever' may not be quite safe enough. Transfus. Med. Hemother. 2004 Jan;31(S-1):2-10.

2. Andreu G, Morel P, Forestier F, Debeir J, Rebibo D, Janvier G, Herve P. Hemovigilance network in France: organization and analysis of immediate transfusion incident reports from 1994 to 1998. Transfusion 2002 Oct;42(10):1356-64.

3. Blajchman MA, Dzik S, Vamvakas EC, Sweeney J, Snyder EL. Clinical and molecular basis of transfusion-induced immunomodulation: summary of the proceedings of a state-of-the-art conference. Transfus. Med. Rev. 2001 Apr;15(2):108-35.

4. Fast LD. Control of immune responses induced by the transfer of allogeneic white blood cells during transfusion. Transfusion 2005 Aug;45(s2):44s-50s.

5. Fast LD, DiLeone G, Li J, Goodrich RP. Functional inactivation of white blood cells by Mirasol treatment. Transfusion 2006 Apr;46(4):642-8.

6. Fast LD, DiLeone G, Edson CM, Purmal A. PEN110 treatment functionally inactivates the PBMNCs present in RBC units: comparison to the effects of exposure to gamma irradiation. Transfusion 2002;42(10):1318-25.

7. Obochi MO, Ratkay LG, Levy JG. Prolonged skin allograft survival after photodynamic therapy associated with modification of donor skin antigenicity. Transplantation 1997 Mar 27;63(6):810-7.

8. Ratkay LG, Waterfield JD, Hunt DW. Photodynamic therapy in immune (non-oncological) disorders: focus on benzoporphyrin derivatives. BioDrugs. 2000 Aug;14(2):127-35.

9. Broady R, Yu J, Levings MK. Pro-tolerogenic effects of photodynamic therapy with TH9402 on dendritic cells. J. Clin. Apher. 2008;23(2):82-91.

10. Heshmati F. Mechanisms of action of extracorporeal photochemotherapy. Transfus. Apheresis. Sci. 2003 Aug;29(1):61-70.

11. Hunt DW, Chan AH. Influence of photodynamic therapy on immunological aspects of disease - an update. Expert Opin. Investig. Drugs 2000;9(4):807-17.

12. Gollnick SO, Liu X, Owczarczak B, Musser DA, Henderson BW. Altered expression of interleukin 6 and interleukin 10 as a result of photodynamic therapy in vivo. Cancer Res. 1997;57(18):3904-9.

13. King DE, Jiang H, Simkin GO, Obochi MO, Levy JG, Hunt DW. Photodynamic alteration of the surface receptor expression pattern of murine splenic dendritic cells. Scand. J. Immunol. 1999;49(2):184-92.

14. Jiang H, Granville DJ, McManus BM, Levy JG, Hunt DW. Selective depletion of a thymocyte subset in vitro with an immunomodulatory photosensitizer. Clin. Immunol. 1999;91(2):178-87.

15. Hunt DW, Jiang H, Granville DJ, Chan AH, Leong S, Levy JG. Consequences of the photodynamic treatment of resting and activated peripheral T lymphocytes. Immunopharm. 1999;41(1):31-44.

16. Jiang H, Granville DJ, North JR, Richter AM, Hunt DW. Selective action of the photosensitizer QLT0074 on activated human T lymphocytes. Photochem. Photobiol. 2002 Aug;76(2):224-31.

17. Trannoy L.L., Lagerberg J.W.M., Dubbelman T.M.A.R., Schuitmaker J.J, Brand A. Positively charged porphyrins: a new series of photosensitizers for sterilization of RBCs. Transfusion 2004 Aug 1;44(8):1186-96.

18. Trannoy LL, Terpstra FG, de Korte D, Lagerberg JWM, Verhoeven AJ, Brand A, van Engelenburg FAC. Differential sensitivities of pathogens in red cell concentrates to Tri-P(4)-photoinactivation. Vox Sang. 2006;91(2):111-8.

19. Trannoy LL, van Hensbergen Y, Lagerberg JW, Brand A. Photodynamic treatment with mono-phenyl-tri-(N-methyl-4-pyridyl)-porphyrin for pathogen inactivation in cord blood stem cell products. Transfusion 2008 Dec;48(12):2629-37.

20. Verduyn W, Doxiadis II, Anholts J, Drabbels JJ, Naipal A, D'Amaro J, Persijn GG, Giphart MJ, Schreuder GM. Biotinylated DRB sequence-specific oligonucleotides. Comparison to serologic HLA-DR typing of organ donors in eurotransplant. Hum. Immunol. 1993 May;37(1):59-67.

21. Vamvakas EC. Meta-analysis of randomized controlled trials of the efficacy of white cell reduction in preventing HLA-alloimmunization and refractoriness to random-donor platelet transfusions. Transfus. Med. Rev. 1998;12(4):258-70.

22. Lakshmanan U, Porter AG. Caspase-4 Interacts with TNF Receptor-Associated Factor 6 and Mediates Lipopolysaccharide-Induced NF-{kappa}B-Dependent Production of IL-8 and CC Chemokine Ligand 4 (Macrophage-Inflammatory Protein-1). J. Immunol. 2007 Dec 15;179(12):8480-90.

23. Lucchi NW, Moore JM. LPS induces secretion of chemokines by human syncytiotrophoblast cells in a MAPK-dependent manner. J. Reprod. Immunol. 2007 Feb;73(1):20-7.

24. Moor AC. Signaling pathways in cell death and survival after photodynamic therapy. J. Photochem. Photobiol. B 2000;57(1):1-13.

25. Fonseca AM, Porto G, Uchida K, Arosa FA. Red blood cells inhibit activation-induced cell death and oxidative stress in human peripheral blood T lymphocytes. Blood 2001;97(10):3152-60.

26. Fialkow L, Wang Y, Downey GP. Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. Free Radic. Biol. Med. 2007 Jan 15;42(2):153-64.

27. Gringhuis SI, Papendrecht-van der Voort EA, Leow A, Nivine Levarht EW, Breedveld FC, Verweij CL. Effect of redox balance alterations on cellular localization of LAT and

downstream T-cell receptor signaling pathways. Mol. Cell Biol. 2002 Jan;22(2):400-11.

28. Legitimo A, Consolini R, Di S, Bencivelli W, Mosca F. Psoralen and UVA light: an in vitro investigation of multiple immunological mechanisms underlying the immunosuppression induction in allograft rejection. Blood Cells Mol. Dis29(1):24-34.

29. Fujihara M, Takahashi TA, Azuma M, Ogiso C, Maekawa TL, Yagita H, Okumura K, Sekiguchi S. Decreased inducible expression of CD80 and CD86 in human monocytes after ultraviolet-B irradiation: its involvement in inactivation of allogenecity. Blood 1996;87(6):2386-93.

30. Verreck FA, de Boer T, Langenberg DM, van der ZL, Ottenhoff TH. Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-gamma- and CD40L-mediated costimulation. J. Leukoc. Biol. 2006 Feb;79(2):285-93.

31. Tzachanis D, Berezovskaya A, Nadler LM, Boussiotis VA. Blockade of B7/CD28 in mixed lymphocyte reaction cultures results in the generation of alternatively activated macrophages, which suppress T-cell responses. Blood 2002;99(4):1465-73.

32. Xu W, Schlagwein N, Roos A, van den Berg TK, Daha MR, van Kooten C. Human peritoneal macrophages show functional characteristics of M-CSF-driven anti-inflammatory type 2 macrophages. Eur. J. Immunol. 2007 Jun;37(6):1594-9.

33. Haugen TS, Nakstad B, Skjonsberg OH, Lyberg T. CD14 expression and binding of lipopolysaccharide to alveolar macrophages and monocytes. Inflammation 1998;22(5):521-32.

34. Palucka KA, Taquet N, Sanchez_Chapuis F, Gluckman JC. Dendritic cells as the terminal stage of monocyte differentiation. J. Immunol. 1998;160(9):4587-95.

35. Fast LD, DiLeone G, Edson CM, Purmal A. Inhibition of murine GVHD by PEN110 treatment. Transfusion 2002;42(10):1326-32.