Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

# The down-regulation of IL1α and IL6, in monocytes exposed to extracorporeal photopheresis (ECP)-treated lymphocytes, is not dependent on lymphocyte phosphatidylserine externalization

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**Keywords**
Annexin V, apoptosis, cytokines, extracorporeal photopheresis, graft versus host disease, monocytes.

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Received: 7 October 2005
Revision requested: 24 October 2005
Accepted: 4 January 2006

doi:10.1111/j.1432-2277.2006.00278.x

## Summary

Extracorporeal photopheresis (ECP) has been successfully used to treat some inflammatory conditions. Following ECP, lymphocytes become apoptotic and untreated monocytes, exposed to post-ECP lymphocytes, reduce proinflammatory cytokine secretion. This study attempted to establish if this monocyte immunosuppression was linked to phosphatidylserine externalization (detected using Annexin V) on the apoptotic lymphocytes. Using density gradient and magnetic separation, lymphocytes were isolated from three cutaneous T-cell lymphoma and nine chronic graft versus host disease (cGvHD) patients pre-ECP and prior to re-infusion (post-ECP). The collected lymphocytes were cultured overnight and Annexin V levels determined. Peripheral blood was taken from the same patient 20 h later and the monocytes were isolated. The 'fresh' monocytes were introduced to each 20 h pre- and post-ECP lymphocyte culture, stimulated with lipopolysaccharide (LPS) and Brefeldin A and subsequently tested for intracellular tumour necrosis factor alpha, interleukin 1 alpha (IL1α), IL1β, IL6 and IL8. For cGvHD patients, the relative levels of IL1α and IL6 were reduced in the untreated, LPS-stimulated monocytes exposed to post-ECP lymphocytes. However, the down-regulation of IL1α and IL6 did not correlate to levels of Annexin V-positive lymphocytes. ECP-treated lymphocytes can reduce the ability of LPS-stimulated monocytes to produce some proinflammatory cytokines; however, this effect is not dependent on phosphatidylserine externalization.

## Introduction

Extracorporeal photopheresis (ECP) is a leucopheresis-based treatment, whereby isolated white cells are exposed to 8-methoxypsoralen (8-MOP) and Ultraviolet (UV) A light. On completion of treatment, the cells are immediately re-infused back to the patient [1]. Although ECP was originally utilized for the treatment of erythrodermic cutaneous T-cell lymphoma (CTCL), many other conditions have subsequently demonstrated a positive response to ECP [1–3]. These conditions often have a proinflammatory aetiology and include rheumatoid arthritis (RA), solid organ rejection, inflammatory bowel disease (IBD) and steroid-refractory acute and chronic graft versus host disease (GvHD) [2,3].

ECP has been demonstrated to directly reduce proinflammatory cytokine production in treated monocytes and lymphocytes [4–7]. More recently, an indirect role for ECP-treated lymphocytes has been proposed, whereby untreated monocytes, exposed to ECP-treated lymphocytes, have down-regulated proinflammatory cytokine production [8].

Following ECP, lymphocytes rapidly become apoptotic and begin to externalize phosphatidlyserine (PS) onto the

 

outer leaf of their cell membrane (flip-flop) [9]. Using flow cytometry, PS externalization can be identified using a flourescene-conjugated Annexin V [10]. PS exposure represents a potent target recognition site for phagocytosis and cells positive for PS are swiftly processed [11]. The process of apoptotic cell phagocytosis, by surrounding antigen-presenting cells (APCs), was originally considered to be a mechanism for the safe removal of potentially dangerous or unnecessary cells without evoking an immune response and subsequent tissue damage [12,13]. However, more recently, removal of apoptotic cells, by APCs, has subsequently been demonstrated to induce immunosuppression [13,14].

This study was designed to determine if the reduction in proinflammatory cytokines, observed in monocytes exposed to ECP-treated lymphocytes, was dependent on the PS externalization observed on apoptotic cells following ECP.

## Patients and methods

### Patients

Twelve patients receiving ECP (three Stage III CTCL and nine extensive, steroid-refractory chronic GvHD (cGvHD)) were tested. For cGvHD, concurrent therapy included steroids and at least one other immunosuppressive agent. This study was conducted in accordance with standards indicated in the 2000 Declaration of Helsinki. Informed consent was obtained from all patients and local ethical approval was granted for the study.

### Photopheresis procedure

ECP was performed using the XTS system (Therakos, Bracknell, UK). The ECP procedure involved isolating white cells, using a six cycle leucopheresis-based system, and exposing them to 8-MOP (UVADEX) (Ben Venue Laboratories, Bedford, OH, USA) and a 1.5 J/cm$^2$ UVA light source. The exposure time was dependent on the volume and haematocrit of the white cell collection bag (approximately 15–60 min). On completion of treatment, all treated cells were re-infused back to the patient immediately.

### Cell preparation

*Day one*

Heparinized samples were taken from each patient immediately pre-ECP and from the white cell collection bag immediately prior to re-infusion (post-ECP). Peripheral blood mononuclear cells (PBMCs) were separated out using density gradient centrifugation (Lymphoprep) (Nycomed, Oslo, Norway) and washed with phosphate buffered saline (containing 1% bovine serum albumin) (PBS). The lymphocytes were positively selected using a magnetic isolation kit (Monocyte isolation kit II; Miltenyi Biotec, Bisley, UK), washed with PBS and added to RPMI medium (Biowhittaker, Verviers, Belgium) containing 10% fetal calf serum, 140 μg/ml streptomycin, 50 μg/ml vancomycin and 1% glutamine to give a final count of $1.0 \times 10^6$/ml. Monocyte contamination of the lymphoid population was determined by adding 5 μl of phycoerythrin-cyanin-5 (PE-CY5)-conjugated anti-CD14 (Beckman-Coulter, High Wycombe, UK). The pre- and post-ECP lymphocytes were incubated overnight (20 h) in the dark at 37 °C.

*Day two*

Heparinized peripheral blood was taken from the same patient 20 h later, on the second day of ECP therapy. Monocytes were negatively selected using a magnetic isolation kit (Monocyte isolation kit II) and washed with PBS. There was a potential risk of using monocytes, which had previously been exposed to ECP during the previous day's treatment. However, their numbers would be small as ECP treats 10–15% of peripheral blood leucocytes and the mean circulation time before a peripheral blood monocyte migrates to the tissues is between 10 and 12 h [3,15]. Furthermore, because the same monocyte population was used for both pre- and post-ECP co-cultures, any observed changes would be comparable. The purity of the monocyte population was determined using 5 μl of PE-CY5 anti-CD14. The monocytes were subsequently added to the 20 h pre- and post-ECP lymphocyte cultures in a ratio of 1:3. Following mixing, the combined cell cultures were stimulated for 5 h with 1 μg/ml LPS and 10 μg/ml Brefeldin A (Sigma-Aldrich, Poole, UK) and incubated in the dark at 37 °C. Preliminary investigations were conducted to establish the influence LPS and Brefeldin A would have on monocyte and lymphocyte viability. Basically, monocytes and lymphocytes were isolated from three cGvHD patients and cultured in either medium only or medium containing LPS and Brefeldin A. No significant increase in Annexin V-positive levels was observed in the LPS/Brefeldin A culture, for either cell population ($P > 0.05$) (data not shown).

### Cell staining

*Lymphocytes*

Prior to the addition of 'day two' monocytes, a sub sample of lymphocytes from the 20 h pre- and post-ECP cultures were tested for apoptosis (PS externalization) using an Annexin V identification kit (Beckman-Coulter). The percentage of Annexin V positive lymphocytes was enumerated using flow cytometry, as previously described [16].



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*Monocytes*

Following stimulation, the cells, within the combined cell cultures, were washed with PBS and the cell count adjusted to $2.0 \times 10^6$/ml. Into 45 μl of re-suspended cells was added 5 μl of PE-CY5 anti-CD14. The cells were incubated in the dark, at room temperature, for 20 min. The cells were immediately washed with PBS and subsequently fixed with 100 μl of 'reagent A' from a cell permeabilization commercial kit (Intrastain) (DAKO-Cytomation, Ely, UK) for 15 min. The cells were washed with PBS again and permeabilized with 100 μl of 'reagent B'. At the permeabilization stage, 20 μl of either phycoerythrin (PE)-conjugated anti-tumour necrosis factor alpha (TNFα), fluorescein isothiocyanate (FITC)-conjugated anti-interleukin 1 alpha (IL1α), PE anti-IL6, FITC anti-IL1β or PE anti-IL8 (R & D Systems, Abingdon, UK) was added. Appropriate isotype controls were performed.

### Flow cytometry

Cells were processed through a Galaxy flow cytometer (DAKO-Cytomation). Alignment and fluorescence were standardized using Alignment beads and Flourospheres (DAKO-Cytomation). A minimum of 30 000 events were gathered for each test. Monocytes were identified using anti-CD14 (PE-CY5) (FL-III) and high side scatter, as previously described [6]. The monocyte region was 'bit-mapped' and from this, cytokine expression was evaluated. 'Quadrant regions' were set on 'dot plots', of FITC (FL-I) and PE (FL-II) expression, using appropriate isotype controls. The quadrant regions were then used to determine the mean fluorescence intensity (MFI) of each cytokine tested. As the anti-cytokine monoclonal antibodies were added in excess, the MFI represented the relative fluorescence expression and thus the relative levels of cytokines produced.

### Statistical evaluation

Using the Shapiro–Wilks *W*-test for non-normality, the levels of monocyte cytokines and the percentage of Annexin V-positive lymphocytes in each cell culture were established to follow a normal distribution ($P > 0.05$) (data not shown). This replicated observations made in our previous study [6–8]. The levels of monocyte cytokines within the pre- and post-ECP cell culture combinations were subsequently compared statistically using the Student paired *t*-test. A *P*-value of <0.05 was regarded as significant. Pearson's correlation analysis between the increase in Annexin V levels of lymphocytes pre- to post-ECP and the fall in IL1α and IL6 MFI of monocytes pre- to post-ECP was also determined for the cGvHD patient cohort.

## Result

The purity of lymphocyte and monocyte populations post-magnetic isolation exceeded 90%. The mean increase in Annexin V-positive lymphocytes, observed 20 h post-ECP, was 33.05%, with a standard error of the mean (SEM) of 3.10.

The mean and SEM of each cytokine MFI, for the untreated monocytes exposed to 20 h cultures of pre- and post-ECP lymphocytes, for both patient groups are displayed in Fig. 1a and b. When comparing the untreated monocytes of CTCL patients, exposed to pre-ECP

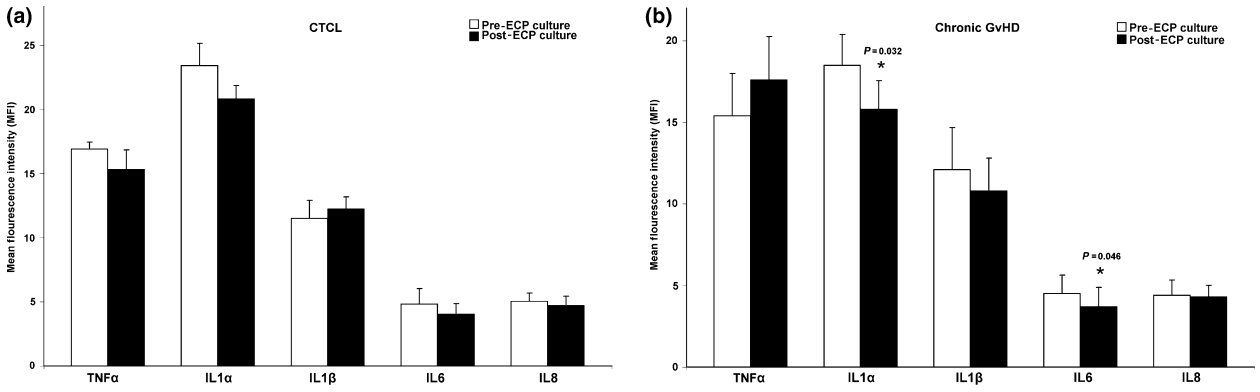


**Figure 1** Monocyte cytokine expression in pre-extracorporeal photopheresis (ECP) and post-ECP cultures. Fresh monocytes were exposed to 20 h cultures of pre- and post-ECP lymphocytes and stimulated with 1 μg/ml lipopolysaccharide and 10 μg/ml Brefeldin A. The relative quantities of tumour necrosis factor alpha (TNFα), interleukin 1 alpha (IL1α), IL1β, IL6 and IL8 were determined in each monocytes population, as a mean florescence intensity (MFI) using a flow cytometry. The mean MFI and standard error of the mean (SEM) (displayed as error bars) of each monocyte cytokine, from the pre-ECP culture (white bars) and post-ECP cultures (black bars), are displayed for the (a) cutaneous T-cell lymphoma (CTCL) and (b) chronic graft versus host disease (Chronic GvHD) patient groups. A significant result is denoted by * and the *P*-value indicated.

lymphocytes, to the culture containing post-ECP lymphocytes, no significant difference was observed for each cytokine tested. However, this could be attributed to the study size, with only three patients tested. Within the cGvHD cohort, a significant reduction in the production of IL1α and IL6, by untreated monocytes, was observed in the post-ECP culture, when compared with the untreated monocytes co-cultured with pre-ECP lymphocytes.

For cGvHD patients, Pearson's correlation analysis was performed between the increase in the percentage of Annexin V-positive lymphocytes, observed in the post-ECP cultures, and the comparable fall of IL1α and IL6 MFI in the untreated monocytes observed in the post-ECP culture. This data is presented in Fig. 2a and b. These results were not significant and thus demonstrated that the reduction of IL1α and IL6, observed in untreated monocytes exposed to post-ECP lymphocytes, was not dependent on the externalization of PS, observed on early apoptotic lymphocytes.

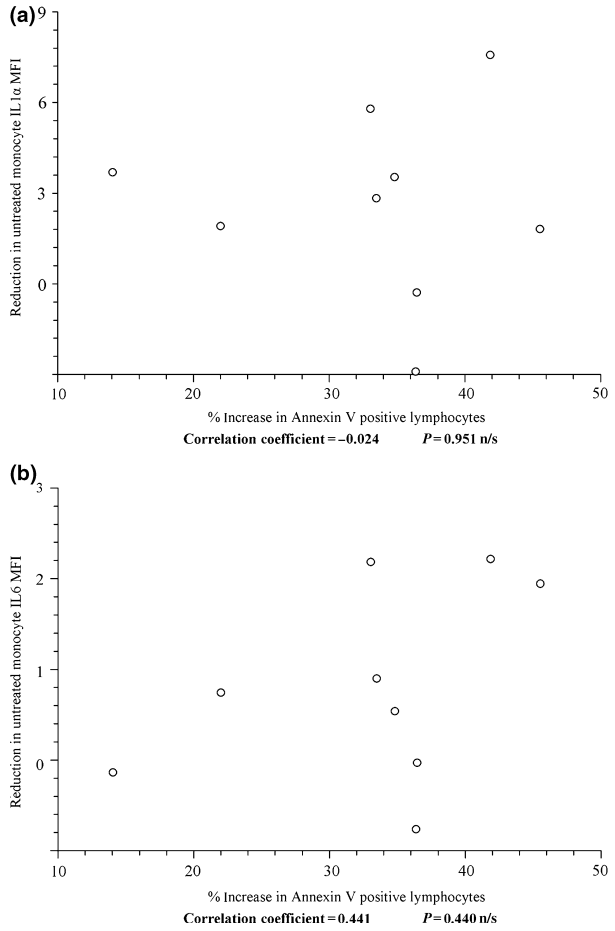


**Figure 2** Correlation between monocyte IL1α and IL6 reduction and increase in Annexin V-positive lymphocytes for chronic graft versus host disease (cGvHD) patients. The reduction in the IL1α and IL6 MFI, of untreated monocytes, for cGvHD patients, observed from pre- to post-ECP cultures and the increase in the percentage of Annexin V-positive lymphocytes, detected from pre- to post-ECP cultures, was correlated. The dot plots display the results of the nine cGvHD patients. Figure 2a demonstrates the comparison of IL1α reduction and Annexin V increase, whilst Fig. 2b displays the comparison between IL6 down-regulation and Annexin V increase. Correlation coefficients and levels of significance are also included. n/s indicates no significance correlation.

## Discussion

ECP is known to induce rapid apoptosis in lymphocytes; to include the majority of treated lymphocytes by 48 h post-exposure [16]. However, the prolonged beneficial effects of ECP therapy still remain elusive. Interaction of apoptotic lymphocytes with APCs has demonstrated an ability to modify APC function, through proinflammatory cytokine reduction and up-regulation of anti-inflammatory secretion [13,14]. More recently, this effect has been observed in the setting of ECP, with ECP-treated lymphocytes capable of reducing production of the proinflammatory cytokines, IL1α, IL1β and IL6 in untreated monocytes [8]. However, in our previous study, the down-regulation of proinflammatory cytokines, in untreated monocytes, was observed when ECP-treated lymphocytes were introduced immediately post-ECP [8]. This study replicated this initial study, but used ECP-treated lymphocytes following 20 h in cell culture, where levels of apoptosis were significantly higher.

As before, this study also demonstrated the ability of ECP-treated lymphocytes to reduce the expression of some proinflammatory cytokines in untreated monocytes. Preliminary investigation had ascertained that this effect was not because of LPS-induced apoptosis; results consistent with other monocytoid test systems [17,18]. However, the down-regulation of monocyte cytokines did not correlate to the levels of Annexin V-positive lymphocytes. Annexin V identifies the externalization of PS, detectable on cells in the early stages of apoptosis [19]. Annexin V-positive cells are swiftly targeted for phagocytosis by APCs [11]. In addition, the down-regulation of cytokine production in the untreated monocytes, exposed to 20 h post-ECP lymphocytes, seemed no more pronounced than that observed in untreated monocytes mixed with *ex vivo* lymphocytes, introduced immediately post-ECP [8]. Although, the different sample population did not allow statistical validation of this point. It would therefore seem that ECP treatment of lymphocytes, independent of PS externalization, has an ability to induce immunosuppression in untreated monocytes.

The reduction in proinflammatory cytokine production, by untreated monocytes, may be dependent of the production of IL10. Although an anti-inflammatory

cytokine, IL10 has been demonstrated to actually enhance the phagocytic properties of monocytes [20]. IL10 has been demonstrated to inhibit the transcription of TNFα, IL1α, IL1β, IL6 and IL8 in LPS-stimulated monocytes [21,22]. Elevated levels of IL10 have been observed in untreated monocytes exposed to ECP-treated lymphocytes [23,24]. However, the lack of any change to TNFα, IL1β or IL8 production, in this study, may support a more direct immunosuppressive response, rather than one mediated by IL-10. Alternatively, the differential reduction in cytokines may be dependent on IL10 levels. Clinical observations, following recombinant IL10 (rhIL10) therapy, have demonstrated that IL10 inhibition of TNFα secretion in LPS-stimulated macrophages is dose-dependent [25].

APCs are a prominent source of the proinflammatory cytokines, which are known to contribute to the aetiology of many inflammatory conditions, including GvHD, IBD and RA [26–28]. Some resolution of acute and chronic GvHD, IBD and RA has been achieved through the targeting of proinflammatory cytokines, such as the anti-TNF agent infliximab [29–33]. For RA, in which most infliximab research has been undertaken, the rapid down-regulation of IL1β and IL6 has also been observed within 4 h of commencing infliximab infusion [33]. Good clinical responses have also been observed in psoriasis patients treated with rhIL10, although RA and IBD demonstrated less positive results following rhIL10 infusions [34]. On each ECP treatment cycle, between 10% and 15% of the total circulating, lymphocytes are treated [3]. The infusion of a large quantity of ECP-treated lymphocytes may significantly contribute to the modulation of cytokine production in these disease states, following APC processing.

ECP induces an indirect immunosuppressive response in untreated monocytes exposed to ECP-treated lymphocytes. However, this process begins prior to demonstrable apoptosis, through PS externalization.

## Conflict of interest

There are no commercial conflicts of interest to declare in connection with this manuscript.

## Acknowledgements

We would like to thank Profs N. Russell, B. Hancock, Drs E. Bessell, J. Davies, J. Snowden, J. Byrne, A. Hunter and G. Cook for referring patients to the Photopheresis Unit at Rotherham General Hospital. Thanks also go to all the patients and nursing staff of the Photopheresis Unit, Rotherham for their assistance with this study.

## References

1. Edelson R, Berger C, Gasparro F, *et al.* Treatment of cutaneous T cell lymphoma by extracorporeal photochemotherapy: preliminary results. *N Engl J Med* 1987; **316**: 297.
2. Rook AH, Suchin KR, Kao DMF, *et al.* Photopheresis: clinical applications and mechanism of action. *J Investig Dermatol Symp Proc* 1999; **4**: 85.
3. Knobler R. Extracorporeal photochemotherapy – present and future. *Vox Sang* 2000; **78** (Suppl. 2): 197.
4. Neuner P, Charvat B, Knobler R, *et al.* Cytokine release by peripheral blood mononuclear cells is affected by 8-methoxypsoralen plus UV-A. *Photochem Photobiol* 1994; **59**: 182.
5. Klosner G, Trautinger F, Knobler R, Neuner P. Treatment of peripheral blood mononuclear cells with 8-Methoxypsoralen plus ultraviolet A radiation induces a shift in cytokine expression from a Th1 to a Th2 response. *J Invest Dermatol* 2001; **116**: 459.
6. Bladon J, Taylor PC. Extracorporeal photopheresis reduces the number of mononuclear cells that produce pro-inflammatory cytokines, when tested ex-vivo. *J Clin Apheresis* 2002; **17**: 177.
7. Bladon J, Taylor PC. Early reduction in number of T cells producing proinflammatory cytokines, observed after extracorporeal photopheresis, is not linked to apoptosis induction. *Transplant Proc* 2003; **35**: 1328.
8. Bladon J, Taylor PC. Lymphocytes treated by extracorporeal photopheresis can down-regulate cytokine production in untreated monocytes. *Photodermatol Photoimmunol Photomed* 2005; **21**: 293.
9. Bladon J, Taylor PC. Extracorporeal photopheresis induces apoptosis in the lymphocytes of cutaneous T cell lymphoma and graft versus host disease patients. *Br J Haematol* 1999; **107**: 707.
10. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cell using fluorescein labelled Annexin V. *J Immunol Methods* 1995; **184**: 39.
11. Fadok. VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* 1992; **148**: 2207.
12. Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980; **68**: 251.
13. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of apoptotic cells. *Nature* 1997; **390**: 350.
14. Albert ML. Death-defying immunity: do apoptotic cells influence antigen processing and presentation? *Nat Rev Immunol* 2004; **4**: 223.
15. Hall R, Malia RG. *Medical Laboratory Haematology*, 1st edn. London: Butterworth & Co (Publishers) Ltd, 1984: **37**.

 

16. Bladon J, Taylor PC. Extracorporeal photopheresis in cutaneous T-cell lymphoma and graft-versus-host disease induces both immediate and progressive apoptotic processes. *Br J Dermatol* 2002; **146**: 59.
17. Suzuki T, Kobayashi M, Isatsu K, *et al.* Mechanisms involved in apoptosis of human macrophages induced by lipopolysaccharide from *Actinobacillus actinomycetemcomitans* in the presence of Cycloheximide. *Infect Immun* 2004; **72**: 1856.
18. Li T, Hu J, Thomas JA, Li L. Differential induction of apoptosis by LPS and taxol in monocytic cells. *Mol Immunol* 2005; **42**: 1049.
19. Verhoven B, Krahling S, Schlegel A, Williamson P. Regulation of phosphatidylserine exposure and phagocytosis of apoptotic T lymphocytes. *Cell Death Differ* 1999; **6**: 262.
20. Buchwald UK, Geerdes-Fenge HF, Vockler J, Ziege S, Lode H. Interleukin-10: effects on phagocytosis and adhesion molecule expression of granulocytes and monocytes in a comparison with prednisolone. *Eur J Med Res* 1999 **4**: 85.
21. Wang P, Wu P, Siegel MI, Egan RW, Ballah MM. IL-10 inhibits transcription of cytokine genes in human peripheral blood mononuclear cells. *J Immunol* 1994: **153**: 811.
22. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* 1991; **174**: 1209.
23. Craciun LI, Stordeur P, Schandene L, *et al.* Increased production of interleukin-10 and interleukin-1 receptor antagonist after extracorporeal photochemotherapy in chronic graft-versus-host disease. *Transplantation* 2002; **74**: 995.
24. Di Renzo M, Rubegni P, Pasqui AL, *et al.* Extracorporeal photopheresis affects interleukin (IL)-10 and IL-12 production by monocytes in patients with chronic graft-versus-host disease. *Brit J Dermatol* 2005; **153**: 59.
25. Tilg H, van Montfrans G, van den Ende A, *et al.* Treatment of Crohn's disease with recombinant human interleukin 10 induces the proinflammatory cytokine interferon gamma. *Gut* 2002 **50**; 191.
26. Reddy P, Ferrara JLM. Immunobiology of acute graft-versus-host disease. *Blood Rev* 2003; **17**: 187.
27. Liote F, Boval-Boizard B, Weill D, Kuntz D, Wautier JL. Blood monocyte activation in rheumatoid arthritis: increased monocyte adhesiveness, integrin expression and cytokine release. *Clin Exp Immunol* 1996; **106**: 13.
28. Podolsky DK. The current future understanding of inflammatory bowel disease.*Best Pract Res Clin Gastroenterol* 2002; **16**: 933.
29. Chiang KY, Abhyankar S, Bridges K, Godder K, Henslee-Downey JP. Recombinant human tumor necrosis factor receptor fusion protein as complementary treatment for chronic graft-versus-host disease. *Transplantation* 2002; **73**: 665.
30. Maini R, St Clair EW, Breedveld F, *et al.* Infliximab (chimeric anti-tumour necrosis factor alpha monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate a randomised phase III trials ATTRACT Study Group. *Lancet* 1999; **354**: 1932.
31. Present DH, Rutgeerts P, Targan S, *et al.* Infliximab for the treatment of fistulas in patients with Crohn's disease. *N Engl J Med* 1999; **340**: 1398.
32. Couriel DR, Hicks K, Giralt S, Champlin RE. Role of tumour necrosis factor- alpha inhibition with inflixiMAB in cancer therapy and haematopoietic stem cell transplantation. *Curr Opin Oncol* 2000; **12**: 582.
33. Feldmann M, Maini RN. Anti TNF alpha therapy of rheumatoid arthritis: what have we learned? *Annu Rev Immunol* 2001; **19**: 163.
34. Asadullah K, Sterry W, Volk HD. Interleukin-10 therapy – review of a new approach. *Pharmacol Rev* 2003; **55**: 241.