

Impact of Alloantigens and Storage-associated Factors on Stimulated Cytokine Response in an In Vitro Model of Blood Transfusion

Andreas E. Biedler, M.D.,* Sven O. Schneider, B.S.,† Ullrich Seyfert, M.D.,‡ Hauke Rensing, M.D.,§ Sasha Grenner, B.S.,† Matthias Girndt, M.D.,|| Inge Bauer, Ph.D.,# Michael Bauer, M.D.**

Background: Transfusion of blood may contribute to immunosuppression in major surgery. The authors assessed the impact of alloantigens and storage on function of peripheral blood mononuclear cells cultured in their physiologic environment.

Methods: Blood units (whole blood, packed erythrocytes) were prepared with or without prestorage leukodepletion and stored for 24–26 days. Blood samples were coincubated with allogeneic fresh blood, autologous, or allogeneic stored blood. Endotoxin-stimulated release of tumor necrosis factor- α (TNF- α) and interleukin 10 (IL-10) was measured after 24 h of culture by enzyme-linked immunosorbent assay.

Results: Coincubation with equal amounts of allogeneic fresh blood showed almost no influence on TNF- α (–12%, not significant) and IL-10 (+11%, not significant) release. Stored allogeneic whole blood resulted in a significant TNF- α depression (–61%) and IL-10 induction (+221%). These effects were diminished but not prevented by prestorage leukodepletion (TNF- α –42%, IL-10 +110%) and required the presence of soluble factors (TNF- α suppression) and cellular components (IL-10 induction). TNF- α decrease and IL-10 increase were in the same order of magnitude (–40%, +134% with, –65%, +314% without leukodepletion) after coincubation with autologous blood. In contrast, allogeneic erythrocytes had only little effects (TNF- α –6%, IL-10 +36%) even at this high transfusion equivalent.

Conclusion: These data suggest that banked whole blood has an immunosuppressive effect that is largely attributable to storage-dependent factors. These factors are partially removed by prestorage leukodepletion, while the contribution of alloantigens is of minor significance. Immunosuppressive effects are least apparent with leukodepleted erythrocytes, suggesting that the presence of plasma during storage is required for the immunosuppressive effect to develop.

SURGICAL trauma is associated with significant impairment of innate and acquired immunity. Transfusion of blood products may contribute to this immunosuppression.¹ Immunosuppression due to blood transfusion may unfavorably affect the postoperative course with an increase in the recurrence rate of malignancies and bacterial infections.^{2,3} Transfusion-associated immunomodulation (TRIM)⁴ seems to depend on degree of contamination with

leukocytes, storage time, and content of cytokines.⁵⁻⁷ Therefore, the reduction of leukocyte content in blood products by prestorage leukodepletion seems to be a valuable concept to prevent TRIM. This concept is not without contradiction,⁸⁻¹¹ and a strong opposition was expressed against universal leukocyte reduction by a large group of American blood bank physicians.¹²

The release of cytokines is a potential pathophysiologic basis for the adverse effects of blood transfusion.¹³⁻¹⁵ Especially the capacity of blood cells to mount a proinflammatory cytokine response on bacterial stimuli may be a valuable assay to detect clinical significant dysfunction of innate immunity.^{16,17} Evidence suggests that a reduced capacity of cultured whole blood obtained after trauma and major surgery to synthesize tumor necrosis factor- α (TNF- α) on lipopolysaccharide stimulation correlates with an unfavorable postoperative course¹⁶⁻¹⁸ and may partially depend on interleukin 10 (IL-10).^{19,20}

We hypothesized that alloantigens and storage-dependent soluble or cell-associated factors might contribute differently to TRIM. This concept may result in clinically significant differences between various blood components such as packed erythrocyte concentrates as opposed to whole blood with respect to immunologic sequelae of blood transfusion. Thus, in the current investigation, we assessed the relative contribution of alloantigens and storage on the ability of peripheral blood mononuclear cells (PBMCs) cultured in their physiologic environment to release the prototypical proinflammatory and antiinflammatory cytokines TNF- α and IL-10.

Materials and Methods

Preparation of Blood Products

The current study was performed according to the Declaration of Helsinki. After we obtained written informed consent, blood components were prepared and samples taken from six healthy blood group O volunteers. The ABO typing was performed by standard hemagglutination techniques (Erytype Biotest, Dreieich, Germany), and the blood of all volunteers was compatible in crossmatch. From five volunteers, whole blood units were collected to serve as autologous blood (*i.e.*, when donor and recipient were identical) or as allogeneic blood (*i.e.*, when donor and recipient were different volunteers but compatible in crossmatch). From the sixth volunteer, a packed erythrocyte concentrate was

* Instructor in Anesthesiology, † Graduate Student, ‡ Resident in Anesthesiology, § Postdoctoral Fellow, ** Assistant Professor, Klinik für Anaesthesiologie und Intensivmedizin, ‡ Associate Professor, Abteilung für Klinische Hämostaseologie und Transfusionsmedizin, || Assistant Professor, Innere Medizin IV, Medizinische Klinik und Poliklinik.

Received from the Klinik für Anaesthesiologie und Intensivmedizin, Abteilung für Klinische Hämostaseologie und Transfusionsmedizin, and Innere Medizin IV, Medizinische Klinik und Poliklinik, Universitätskliniken des Saarlandes, Homburg/Saar, Germany. Submitted for publication November 6, 2001. Accepted for publication June 11, 2002. Support was provided solely from institutional and/or departmental sources.

Address reprint requests to Dr. Bauer: Klinik für Anaesthesiologie und Intensivmedizin, Universitätskliniken des Saarlandes, 66421 Homburg/Saar, Germany. Address electronic mail to: aimbau@med-rz.uni-sb.de. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

obtained. All blood units were collected according to Good Clinical Practice guidelines under assistance of the local blood bank and were stored, if required, in the blood bank under standard conditions for 24–26 days at +4°C. Samples from blood bags were drawn by using a 40- μ m blood filter (Paed Set 1; Wex Filtertechnik, Rotenburg, Germany) to remove aggregates.

The following blood components-preparations were used. Fresh whole blood was drawn from the volunteers into commercially available citrated plastic tubes (Monovette; Sarstedt, Nümbrecht, Germany). Stored whole blood units (250 ml) were collected using an automatic mixing and weighing device (Febra, Tucson, AZ) into a multiple bag system (Fresenius NPBI, Dreieich, Germany) containing 35 ml citrate, phosphate dextrose, and adenine solution as anticoagulant and stabilizer. Leukodepleted whole blood units were obtained by separating half of the volume of the whole blood unit at room temperature into a satellite bag using the integrated in-line leukocyte filter (Compoline; Fresenius NPBI) within 1 h after donation (prestorage leukodepletion), allowing direct comparison of units with or without prestorage leukodepletion from one donor. A standard buffy-coat-depleted and prestorage leukodepleted group O packed erythrocyte concentrate was obtained by drawing 450 ml of whole blood in a quadruple bag in top and bottom configuration (Fresenius NPBI) with 70 ml citrate, phosphate dextrose, and adenine solution as the anticoagulant. Immediately after collection, the blood was filtered through the integrated in-line filter (Compoline; Fresenius NPBI), centrifuged at 5,000g for 15 min using an automatic system for blood component preparation (Cryofuge 6000I; Heraeus-Kendro, Osterode, Germany), and stored in 110 ml PAGGS-mannitol as the stabilizer.

Additional Experiments to Address the Role of Cellular and Humoral Components on the Observed Effects of Stored Whole Blood

To further characterize the contribution of cellular components as opposed to soluble factors present in stored whole blood, in an additional series of experiments a whole blood unit stored for 26 days under blood bank conditions was centrifuged at 3,400g for 15 min (Cryofuge 6000i), and the supernatant and cellular fractions were separately studied with respect to their modulatory effect on stimulated cytokine release in a vol:vol ratio of 1:1.

Coculture Assays, Ex Vivo Stimulation Experiments, and Processing of Samples

As an *in vitro* model of blood transfusion, fresh blood, stored whole blood with or without prestorage leukodepletion, and packed erythrocyte concentrate were added to whole blood of a recipient and subjected to coculture. The following blood components were added: (1) fresh blood of allogeneic donor; (2) stored whole blood of allogeneic donor; (3) prestorage leukodepleted whole blood of allogeneic donor; (4)

stored autologous whole blood; (5) prestorage leukodepleted autologous whole blood; and (6) allogeneic packed erythrocyte concentrate of donor.

Venous samples ($n = 5$ each) serving as recipient were drawn immediately before the subsequent *in vitro* investigation into commercially available citrated plastic tubes (Monovette) and coincubated with and without equal amounts (vol:vol ratio 1:1) of one of the components 1–6 specified above. The components obtained from donors (1–6) were also subjected to *ex vivo* lipopolysaccharide stimulation in the absence of blood of recipient to determine their direct contribution to the cytokines measured in the supernatants of the coculture assays. Thus, the anticipated value for lipopolysaccharide-stimulated TNF- α and IL-10 release of the coculture assays was the arithmetic mean of the value obtained for pure recipient and pure donor samples after stimulation with lipopolysaccharide in the culture assay.

To obtain information on the dose-dependent effects of transfusion, venous blood was also coincubated with increasing amounts of selected blood components. These additional cocultures were established containing 25% and 75% of venous blood together with 75% and 25% of fresh allogeneic whole blood, stored allogeneic whole blood, and stored allogeneic packed erythrocyte concentrate, respectively ($n = 5$ each), leading to additional vol:vol ratios of 1:3 and 3:1.

Ex Vivo Endotoxin Stimulation

The immunomodulatory effects of the various blood components were studied in an adaptation of the whole blood culture system, originally described by Wilson *et al.*²¹ to study *ex vivo* PBMC function. We have used this assay with minor modifications previously to analyze immunomodulatory effects of anesthetics²² as well as immunomodulatory effects of plasma obtained from patients undergoing coronary artery bypass grafting.²⁰

After addition of heparin (2 units/ml; tested for endotoxin < 5 pg/ml) and recalcification (by adding CaCl₂ in equimolar amounts to neutralize the citrate ions), aliquots of blood with or without addition of the blood components under study were subjected to culture in a 24-well plate in a humidified atmosphere with 5% carbon dioxide at 37°C for 24 h. Each coculture of blood of a recipient with addition of blood components defined above (1–6) was stimulated by addition of 1 μ g/ml lipopolysaccharide (*Escherichia coli* O111:B4), *i.e.*, a dose that induces a maximum release of both cytokines studied. At 24 h after onset of culture, the supernatant was collected from the wells and stored immediately at –80°C until it was assayed for cytokines.

Measurement of Cytokines Released during Culture

Samples were assessed for TNF- α and IL-10 concentrations by means of commercially available standard en-

zyme-linked immunosorbent assay kits (h-Tumor Necrosis Factor- α ELISA and h-Interleukin-10 ELISA; Roche Molecular Diagnostics, Mannheim, Germany). Samples were thawed at room temperature and were diluted 1:20 with the provided diluent to stay within the linear range of the assay; controls for both cytokines studied provided with the kits were measured routinely with each assay as we have reported previously.²² Calculated intraassay and interassay coefficients of variance were 11.4% (14.4%) for TNF- α and 9.7% (7.0%) for IL-10, respectively. The minimal detectable concentrations for both cytokines were estimated from the average optical density reading of the zero standard plus 2 SDs and were approximately 3 pg/ml for TNF- α and 1 pg/ml for IL-10.

Statistical Analysis

Data are reported as median, 25th and 75th percentile, and range. Statistical differences were determined after confirming normal distribution and equal variance (Kolmogorov-Smirnow and Levene-Mediane tests) by one-way repeated-measures analysis of variance followed, if significant, by *post hoc* Student-Newman-Keuls test. Data obtained in the coculture assays were normalized to the proportionate values expected from cytokine concentrations obtained in the pure fractions subjected to coculture, *i.e.*, for 1:1 mixtures the arithmetic mean of values obtained for both donors was expected to reflect 100%. Other dilutional values were calculated from the cytokine response of venous blood, the respective added blood product, and the respective ratio of both. This approach allowed for the correction of changes in the absolute number of cells producing cytokines derived in variable amounts from different donors. This is particularly important since the cytokine response is known to exhibit substantial interindividual variability as a result of promoter polymorphisms.²³

Statistical tests were performed using the SigmaStat software package (Jandel, Erkrath, Germany). All *P* values reported are two-sided and are considered statistically significant at $P \leq 0.05$.

Results

Lipopolysaccharide-stimulated Release of Tumor Necrosis Factor α and Interleukin 10 by Fresh Whole Blood and the Different Blood Components Used in Coculture Assays

Lipopolysaccharide-stimulated cytokine release was 31,733 pg/ml (range, 25,600–39,066 pg/ml) for TNF- α and 377 pg/ml (range, 327–450 pg/ml) for IL-10 in fresh venous blood of recipients. All stored blood components investigated presented lipopolysaccharide-stimulated TNF- α and IL-10 concentrations significantly lower than those obtained in fresh venous blood. Mean concentrations of TNF- α and IL-10 were 244 pg/ml (range, 170–

300 pg/ml) and 13 pg/ml (range, 12–14 pg/ml) in stored whole blood, 40 pg/ml (range, 30–45 pg/ml) and 9 pg/ml (range, 8–11 pg/ml) in prestorage leukodepleted whole blood, and 50 pg/ml ($n = 1$) and 10 pg/ml ($n = 1$) in the stored packed erythrocyte concentrate after 24-h incubation with lipopolysaccharide.

Effect of the Different Blood Components on the Lipopolysaccharide-stimulated Tumor Necrosis Factor- α and Interleukin 10 Release

Coincubation of blood of recipients with equal amounts of fresh allogeneic blood (donor) failed to significantly influence lipopolysaccharide-stimulated TNF- α release (median, 21,024 pg/ml; range, 175–23,446 pg/ml; fig. 1A). In contrast, cultures with allogeneic whole blood from the identical donor stored for 24–26 days decreased the TNF- α release significantly (median, 6,713; range, 4,013–8,486 pg/ml; fig. 1A). This effect of stored blood was partially and variably prevented by prestorage leukodepletion with in-line filtration (median, 5,395 pg/ml; range, 3,449–18,122 pg/ml; fig. 1B); the inhibitory effect of prestorage leukofiltrated allogeneic whole blood did not longer reach statistical significance. Similar results were obtained for autologous whole blood: undepleted stored autologous whole blood (*i.e.*, recipient and donor were identical) inhibited the TNF- α response significantly and to a similar extent as observed with allogeneic whole blood (median, 5,702 pg/ml; range, 1,990–8,541 pg/ml; fig. 1C). This inhibitory effect of autologous stored whole blood was also partially prevented (median, 9,479 pg/ml; range, 4,235–13,604 pg/ml) by prestorage leukodepletion (fig. 1D); similar to what was observed in the case of allogeneic whole blood, this effect of leukodepletion was variable, and the inhibitory effect of leukodepleted autologous blood on stimulated TNF- α release no longer reached statistical significance.

In contrast to what was observed for stored autologous and allogeneic whole blood, coincubation with equal amounts of allogeneic packed erythrocyte concentrate had no significant influence on the lipopolysaccharide-stimulated TNF- α release (median, 9,900 pg/ml; range, 8,712–16,280 pg/ml; fig. 1E) even at this high transfusion equivalent.

As seen with TNF- α release, the lipopolysaccharide-induced IL-10 concentration remained unaffected by coincubation with equal amounts of allogeneic fresh blood (median, 377 pg/ml; range, 344–475 pg/ml; fig. 1A). While stored blood had a suppressive effect on the release of the prototypical proinflammatory cytokine TNF- α , its presence in the coculture assays had a permissive effect on the release of the antiinflammatory cytokine IL-10. A significant increase of lipopolysaccharide-induced IL-10 release was found with allogeneic stored whole blood after 24–26 days of storage (median, 520 pg/ml; range, 499–742 pg/ml; fig. 1A), which was attenuated by prestorage leukofiltration (median, 369 pg/ml; range, 363–449 pg/ml; fig. 1B), and the increase

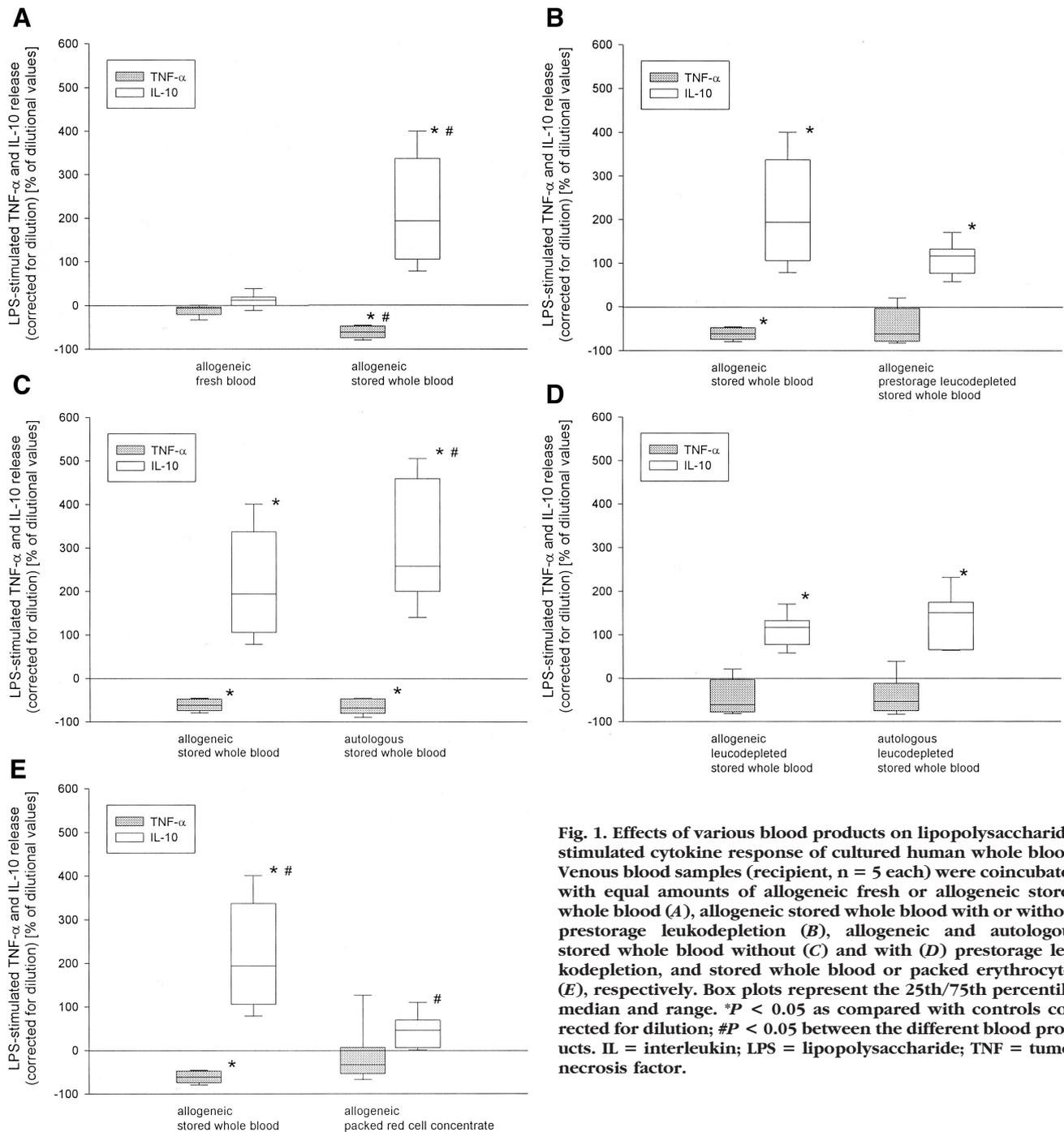


Fig. 1. Effects of various blood products on lipopolysaccharide-stimulated cytokine response of cultured human whole blood. Venous blood samples (recipient, $n = 5$ each) were coincubated with equal amounts of allogeneic fresh or allogeneic stored whole blood (A), allogeneic stored whole blood with or without prestorage leukodepletion (B), allogeneic and autologous stored whole blood without (C) and with (D) prestorage leukodepletion, and stored whole blood or packed erythrocytes (E), respectively. Box plots represent the 25th/75th percentile, median and range. * $P < 0.05$ as compared with controls corrected for dilution; # $P < 0.05$ between the different blood products. IL = interleukin; LPS = lipopolysaccharide; TNF = tumor necrosis factor.

in IL-10 in the presence of stored leukodepleted blood no longer reached statistical significance. Similar results were obtained for autologous components, *i.e.*, autologous stored whole blood led to a remarkable increase in lipopolysaccharide-induced IL-10 release (median, 606 pg/ml; range, 567–887 pg/ml; fig. 1C) that was significantly reduced by prestorage leukodepletion (median, 432 pg/ml; range, 370–445 pg/ml; fig. 1D).

In contrast to stored whole blood, the stored allogeneic packed erythrocyte concentrate exhibited no significant influence on lipopolysaccharide-stimulated IL-10

release (median, 288 pg/ml; range, 250–319 pg/ml; fig. 1E) at a vol:vol ratio of 1:1.

Dose-Response Characteristics of the Observed Effects of Different Blood Components

Coincubation with increasing amounts of fresh allogeneic blood and allogeneic packed erythrocyte concentrate were without a specific effect on lipopolysaccharide-stimulated TNF- α release in all vol:vol ratios subjected to coculture (3:1, 1:1, 1:3) when results were corrected for admixture (figs. 2A and B). In contrast,

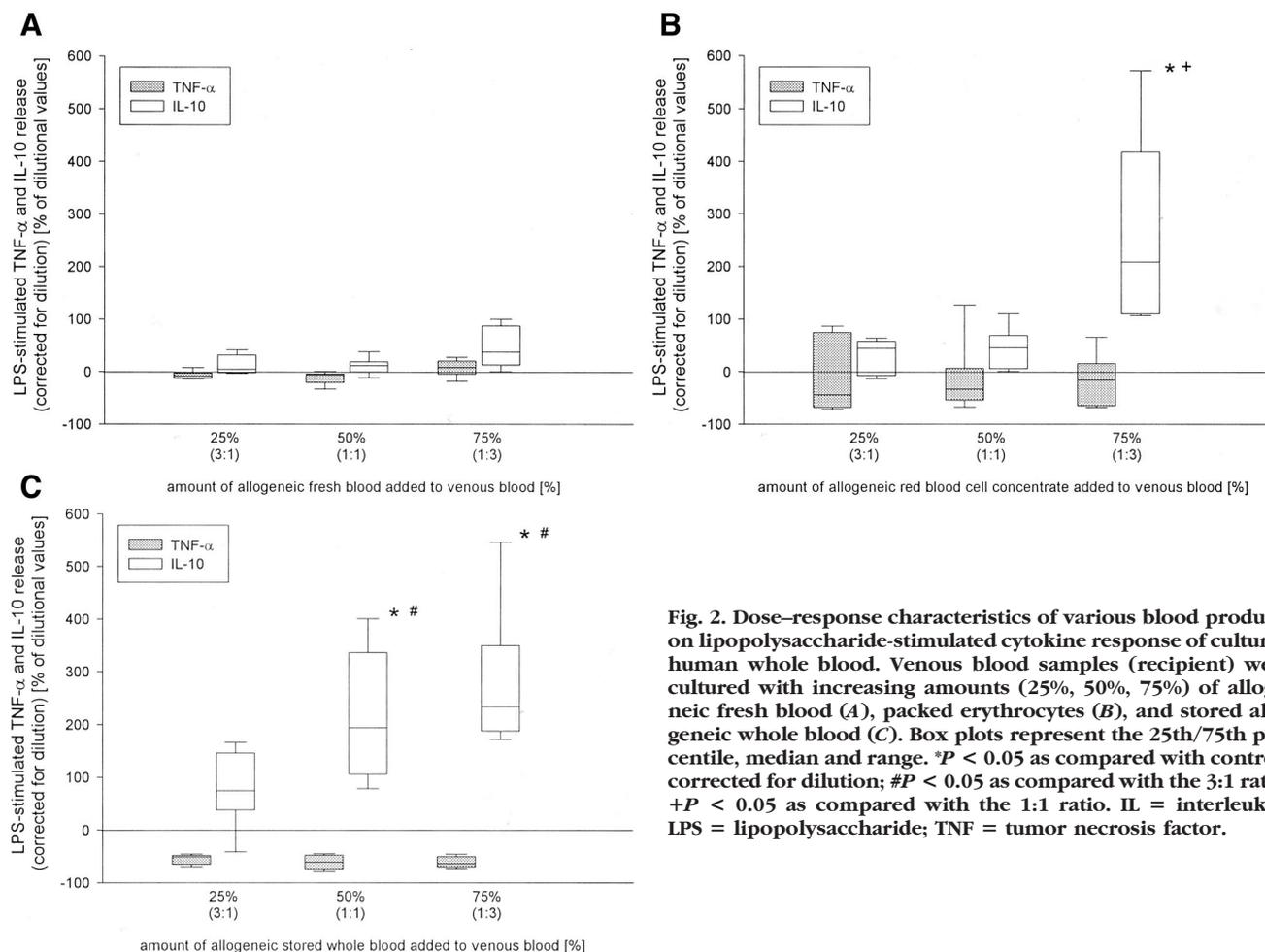


Fig. 2. Dose–response characteristics of various blood products on lipopolysaccharide-stimulated cytokine response of cultured human whole blood. Venous blood samples (recipient) were cultured with increasing amounts (25%, 50%, 75%) of allogeneic fresh blood (A), packed erythrocytes (B), and stored allogeneic whole blood (C). Box plots represent the 25th/75th percentile, median and range. * $P < 0.05$ as compared with controls corrected for dilution; # $P < 0.05$ as compared with the 3:1 ratio; + $P < 0.05$ as compared with the 1:1 ratio. IL = interleukin; LPS = lipopolysaccharide; TNF = tumor necrosis factor.

stored whole blood suppressed TNF- α release at all vol:vol ratios significantly after correction for dilution (3:1, median 10,277 and range 9,625–11,739 pg/ml; 1:1, median 6,713 and range 4,013–8,486 pg/ml; 1:3, median 3,276 and range 3,084–3,460 pg/ml). This inhibitory effect of stored blood on stimulated TNF- α release was, however, without a dose-effect relation (fig. 2C).

The lipopolysaccharide-stimulated IL-10 release was unaffected by allogeneic fresh blood when corrected for effects of admixture in all vol:vol ratios (fig. 2A). However, at odds to what was observed for TNF- α release, addition of the packed erythrocyte concentrate led to a significant increase in lipopolysaccharide-stimulated IL-10 release at the high vol:vol ratio of 3:1, whereas the lower vol:vol ratios failed to affect stimulated IL-10 (fig. 2B). As seen with TNF- α , stored whole blood led to a significant increase in IL-10 in all vol:vol ratios, and this increase appeared to be dose-dependent (fig. 2C).

Contribution of Soluble and Cellular Factors to the Immunomodulatory Effect of Stored Whole Blood

To further address the relative role of humoral as opposed to cellular factors mediating the immunosuppressive effect of stored whole blood, additional exper-

iments were conducted in which supernatant and cellular fractions were separated by centrifugation and were incubated selectively with blood of a recipient (fig. 3). These experiments revealed a discriminate role for supernatant and cellular components in mediating the immunosuppressive effect of stored blood. While supernatant mediated the inhibitory effect of stored whole blood on lipopolysaccharide-stimulated TNF- α response (median, 1,579 pg/ml; range, 1,349–2,315 pg/ml) significantly, it barely effected the stimulated IL-10 release (median, 426 pg/ml; range, 324–540 pg/ml). In contrast to what was observed with soluble components, presence of the cellular fraction significantly increased both the TNF- α response (median, 17,497 pg/ml; range, 15,382–18,964 pg/ml) and, most notably, the IL-10 release (median, 932 pg/ml; range, 787–2,588 pg/ml). Thus, the net inhibitory effect of whole blood on stimulated TNF- α response depends on the presence of soluble factors and is more prominent than the permissive effect on stimulated TNF- α response of the cellular fraction. However, the increased production of IL-10 mediated by stored whole blood seems to exclusively require the presence of the cellular fraction.

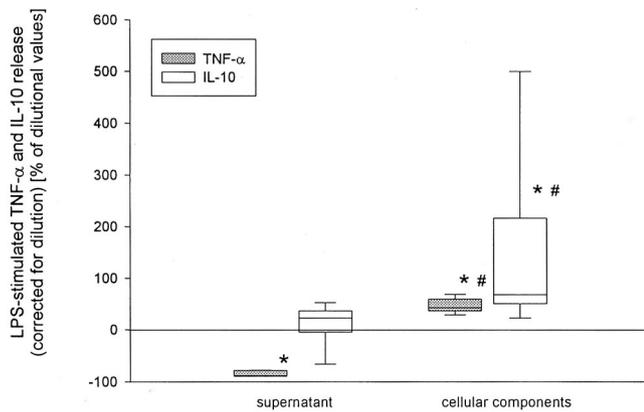


Fig. 3. Effects of soluble factors and cellular components separated from stored whole blood on lipopolysaccharide-stimulated cytokine response. Stored allogeneic whole blood of a donor was separated into soluble and cellular factors by centrifugation. Each fraction was coincubated with equal amounts of fresh blood of a recipient ($n = 5$). Box plots represent the 25th/75th percentile, median and range. * $P < 0.05$ as compared with controls corrected for dilution; # $P < 0.05$ between soluble and cellular fraction. IL = interleukin; LPS = lipopolysaccharide; TNF = tumor necrosis factor.

Discussion

In the current study, we investigated the influence of autologous and allogeneic transfusion on lipopolysaccharide-stimulated cytokine production in an *in vitro* transfusion model using cultured human whole blood. We found an immunosuppressive effect of transfusion, as indicated by changes in stimulated release of the prototypical proinflammatory cytokine TNF- α and increased release of the antiinflammatory IL-10 in the presence of stored whole blood in the coculture system. These effects could be reduced but not eliminated by prestorage leukofiltration and were barely apparent when leukodepleted packed erythrocytes were subjected to coculture with blood of a recipient. Of note, no substantial differences were detected between allogeneic and autologous blood products.

Based on previous results confirming an influence of operative trauma and perioperative care on the immune system, we used an *in vitro* blood transfusion model to evaluate particularly the contribution of transfusion to the observed dysfunction of innate immunity. More specifically, factors that have been proposed to mediate these effects, *i.e.*, alloantigens and storage, were studied in detail. This *ex vivo* model allows study of PBMC functions in their unaffected *milieu interne*, because many of the regulatory mechanisms remain intact.²¹ Since the immunomodulatory effects of surgery are of outstanding significance in the context of nosocomial infection, we selected lipopolysaccharide stimulation as a model of sequential gram-negative challenge. Stimulated cytokine production provides information on the ability of the recipient's immune system to respond to infectious stimuli, for example, as this would be the case

in nosocomial pneumonia. The cytokines assessed were TNF- α and IL-10 as prototypical proinflammatory (TNF- α) and antiinflammatory (IL-10) cytokines. IL-10 promotes Th2-type immune response and has been described as the major inhibitor of T-helper type-1 cell functions.²⁴ Data obtained in experimental and clinical studies suggest that the balance of monocytic proinflammatory and antiinflammatory cytokines is crucial for the immune response and eventual development of host defense failure.^{18,25} Patients with a depression of lipopolysaccharide-stimulated TNF- α release together with an increase in IL-10 release after major surgery are known to show an unfavorable postoperative course.^{16,17} Consistent with a potential contribution of blood transfusion to the observed perioperative dysfunction of innate immunity, a similar pattern of depression of proinflammatory and increase of antiinflammatory cytokine release was observable in our model. This pattern of enhanced production of IL-10 while TNF- α is markedly suppressed reflects reprogramming rather than a general depression of the PBMC cytokine response and is in agreement with previously reported *in vitro*²⁶ and *in vivo*²⁷ data. It is therefore conceivable that blood transfusion may exert its immunosuppressive effect, in part, by stimulating Th2-derived IL-10 production, which may be pivotal in septic or severely injured patients with their already impaired proinflammatory immune response.

In clinical investigations, significant immunosuppression as a result of transfusion was suggested to contribute to cancer recurrence and susceptibility to postoperative infection.^{2,3,8} One of the factors suggested to be responsible for this immunosuppression in the context of blood transfusion are alloantigens. Clinical studies comparing transfusion of allogeneic blood and autologous blood could show fewer infections, less impairment of immune functions, and even reduced hospital stay when patients received autologous instead of allogeneic transfusion.^{5,28-31} However, autologous blood products appear not to be immunologically inert. Changes in cytokine production and composition of T-cell subpopulations were seen also in patients receiving autologous transfusion.^{32,33} The current *in vitro* investigation suggests that effects of banked whole blood on PBMC function are similar for autologous and allogeneic transfusions, consistent with an effect of storage since both showed a comparable decrease in TNF- α release and increase in IL-10 release on lipopolysaccharide stimulation.

It has been suggested that the leukocyte content in blood components may be the major determinant of transfusion-induced adverse effects.³⁴ However, the current investigation indicates that not leukocytes *per se* appear to be responsible, since fresh allogeneic blood with normal leukocyte blood count had no significant effect on IL-10 and TNF- α release. Effects on TNF- α and IL-10 release were found in the current study only in the

presence of stored blood products. Leukocytes undergo degeneration and apoptosis during refrigerated storage. Most leukocytes are apoptotic after 4 weeks.³⁵ Apoptotic cells that are present in transfused blood will not induce immunosuppression through production of immunologically active substances. Nevertheless, MHC-peptide complexes that are still present on apoptotic cells have been suggested to act as T-cell receptor antagonists and induce inhibition of T-cell functions.³⁶ However, in the current investigation there was no difference in the immune response to lipopolysaccharide after addition of autologous or allogeneic stored blood, therefore suggesting that HLA antigens expressed on leukocytes or their fragments appear to be of limited significance for the alterations in a key monocyte function, *i.e.*, release of the cytokines TNF- α and IL-10 observed. Obviously, factors related to the storage of blood products are of outstanding significance for the observed effects. Evidence suggests that with the degeneration of leukocytes, leukocyte-derived bioactive substances such as histamine, eosinophil cationic protein, eosinophil protein X, and myeloperoxidase accumulate extracellularly in blood components that are all known to play a significant role in immunosuppression and tissue damage.³⁷⁻³⁹ Because the accumulation of bioactive substances is independent of autologous or allogeneic origin, this assumption that leukocyte-derived bioactive substances are a major source for TRIM, could also explain why several investigations failed to document a difference between allogeneic and autologous blood transfusion.^{9,27,40}

Based on the assumption that leukocyte-derived substances are responsible for the immunomodulation of blood transfusion, prestorage leukofiltration of blood products appears to be a valuable approach to prevent TRIM. There are several clinical investigations that would be consistent with a favorable effect of leukodepletion on tumor recurrence and the occurrence of postoperative infectious complications.⁶ In our investigation, there was a significant effect of prestorage leukofiltration on stimulated cytokine release. Both TNF- α suppression and IL-10 increase seen with unfiltered whole blood were attenuated. However, prestorage leukofiltration did not restore the lipopolysaccharide responsiveness of cultured whole blood completely. This is in agreement with previous investigations, where leukofiltration did not lead to complete restoration of storage time-dependent TNF- α suppression^{41,42} and would explain why immunomodulatory effects were described also after transfusion of leukocyte-depleted blood.¹⁰ The remaining immunomodulatory effect of blood transfusion even with prestorage leukodepletion suggests that either the few remaining leukocytes mediate these effects or that other factors than leukocyte-derived mediators are contributing to the immunomodulatory effects of transfusion. For instance, it was shown that erythrocytes might also play a role in storage time-dependent immunosuppres-

sion, since erythrocyte membrane phospholipids were shown to activate macrophage derived prostaglandins, which are potent immune regulatory factors.^{43,44}

To discriminate whether soluble components, *i.e.*, plasma, or cellular components are responsible for the immunomodulation observed with stored blood, we investigated both fractions, *i.e.*, supernatant and cellular components, selectively. Interestingly, there was a remarkable difference in the lipopolysaccharide-stimulated cytokine release after coincubation with these fractions with respect to decreased TNF- α and increased IL-10 response: while supernatant induced a depression of TNF- α in similar order of magnitude as observed with whole blood, the increase in IL-10 was found to be very moderate in the presence of soluble factors as compared with whole blood. In contrast, a substantial increase in stimulated IL-10, which was in the same order of magnitude as observed with whole blood, was associated with the addition of cellular components. This implicates that cellular components are mainly responsible for the increased release of IL-10 after addition of stored blood, whereas plasma factors are mainly responsible for depression of TNF- α . However, our data would suggest that simultaneous presence of both cellular components and plasma during storage is required for development of the complete immunosuppressive effect observed with stored whole blood. This is further supported by the very moderate immunomodulatory effect that we found with prestorage leukodepleted erythrocyte concentrates, where almost all plasma was eliminated during the production process. Even at a vol:vol ratio of 1:1, erythrocytes had neither suppressive effects on stimulated TNF- α nor a permissive effect on stimulated IL-10. Only after coincubation of erythrocytes in a vol:vol ratio of 3:1 corresponding to a transfusion equivalent of more than 10 units of packed erythrocytes in a normal adult, a significant increase in IL-10 but no decrease in TNF- α release was observed. Thus, it is conceivable that the immunologic effects of leukocyte-reduced blood or autologous blood components other than packed erythrocytes may reflect a possible explanation for the contradictory results reported for nonhemolytic detrimental effects of blood transfusion.

References

1. Klein HG: Immunomodulatory aspects of transfusion. *ANESTHESIOLOGY* 1999; 91:861-5
2. Busch ORC, Hop WCJ, Hoynck van Papendrecht MAW, Marquet RL, Jeekel J: Blood transfusion and prognosis in colorectal cancer. *N Engl J Med* 1993; 328:1372-6
3. Vamvakas E, Moore SB: Blood transfusion and postoperative septic complications. *Transfusion* 1994; 34:714-27
4. Blajchman MA: Allogeneic transfusion, immunomodulation and postoperative bacterial infection: Do we have the answers yet? *Transfusion* 1997; 37:121-5
5. Innerhofer P, Luz G, Spötl L, Hobisch-Hagen P, Schobersberger W, Fischer M, Nussbaumer W, Lochs A, Irschick E: Immunologic changes after transfusion of autologous or allogeneic buffy coat-poor versus white cell-reduced blood to patients undergoing arthroplasty. I. Proliferative T-cell responses and the balance of helper and suppressor T cells. *Transfusion* 1999; 39:1089-96

6. Jensen LS, Kissmeyer-Nielsen P, Wolff B, Qvist N: Randomized comparison of leucocyte-depleted versus buffy-coat-poor blood transfusion and complications after colorectal surgery. *Lancet* 1996; 348:841-5
7. Weisbach V, Wanke C, Zingssem J, Zimmermann R, Eckstein R: Cytokine generation in whole blood, leucocyte-depleted and temporarily warmed red blood cell concentrates. *Vox Sang* 1999; 76:100-6
8. Brand A, van de Watering LM, Claas FH: Clinical significance of leukoreduction of blood components. *Vox Sang* 2000; 78(suppl 2):227S-9S
9. Busch OR, Hop WC, Marquet RL, Jeekel J: Autologous blood and infections after colorectal surgery. *Lancet* 1994; 343:668-9
10. Houbiers JG, van de Velde CJ, van de Watering LM, Hermans J, Schreuder S, Bijnen AB, Pahlplatz P, Eeftink Schattenkerk M, Wobbles T, de Vries JE, Klementsitsch P, van de Maas AH, Brand A: Transfusion of red cells is associated with increase incidence of bacterial infection after colorectal surgery: A prospective study. *Transfusion* 1997; 37:126-34
11. Vamvakas EC, Blajchman MA: Universal WBC reduction: The case for and against. *Transfusion* 2001; 41:691-712
12. Thurer RL, Luban NL, AuBuchon JP, Silver H, McCarthy IJ, Dzik S, Stowell CP, Moore SB, Vamvakas EC, Armstrong W, Kanter MH, Jeter E, Becker J, Higgins M, Gale S, Kleinman S, Marshall CS, Newman R, Ocariz JA, Blackall D, Petz LD, Toy P, Oberman H, Siegel DL, Price TH, Slichter SJ: Universal WBC reduction. *Transfusion* 2000; 40:751-2
13. McBride WT, Armstrong MA, McBride SJ: Immunomodulation: An important concept in modern anaesthesia. *Anaesthesia* 1996; 51:465-73
14. Davenport RD, Strieter RM, Kunkel SL: Red cell ABO incompatibility and production of tumor necrosis factor-alpha. *Br J Haematol* 1991; 78:540-4
15. Grunenberger R, Krüger J: Analysis of cytokine profiles of stored CPDA-1 whole blood. *Infusionsther Transfusionsmed* 1995; 22:292-4
16. Heagey W, Hancan C, Nieman K, Cohen M, Richardson C, Rodriguez JL, West MA: Impaired ex vivo lipopolysaccharide-stimulated whole blood tumor necrosis factor production may identify "septic" intensive care unit patients. *Shock* 2000; 14:271-7
17. Ziegenfuß T, Wanner GA, Grass C, Bauer I, Schüder G, Kleinschmidt S, Menger MD, Bauer M: Mixed agonistic-antagonistic cytokine response in whole blood from patients undergoing abdominal aortic aneurysm repair. *Intensive Care Med* 1999; 25:279-87
18. Ertel W, Kremer JP, Kenney J, Steckholzer U, Jarrar D, Trentz O, Schildberg FW: Downregulation of proinflammatory cytokine release in whole blood from septic patients. *Blood* 1995; 85:1341-7
19. Randow F, Syrbe U, Meisel C, Krausch D, Zuckermann H, Platzer C, Volk HD: Mechanism of endotoxin desensitization: Involvement of interleukin-10 and transforming growth factor. *J Exp Med* 1995; 181:1887-92
20. Grundmann U, Rensing H, Adams HA, Falk S, Wendler O, Ebinger N, Bauer M: Endotoxin desensitization of human mononuclear cells after cardiopulmonary bypass: Role of humoral factors. *ANESTHESIOLOGY* 2000; 93:359-69
21. Wilson BMG, Severn A, Rapson NT, Chana J, Hopkins P: A convenient human whole blood culture system for studying the regulation of tumor necrosis factor release by bacterial lipopolysaccharide. *J Immunol Methods* 1991; 139:233-40
22. Larsen B, Hoff G, Wilhelm W, Buchinger H, Wanner GA, Bauer M: Effect of intravenous anesthetics on spontaneous and endotoxin-stimulated cytokine response in cultured human whole blood. *ANESTHESIOLOGY* 1998; 89:1218-27
23. McGuire W, Hill AV, Allsopp CE, Greenwood BM, Kwiatkowski D: Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. *Nature* 1994; 371:508-10
24. Del Prete G, Maggi E, Romagnani S: Human Th1 and Th2 cells: Functional properties, mechanisms of regulation, and role in disease. *Lab Invest* 1994; 70:299-306
25. Faist E, Hartl WH, Bauer AE: Immune mechanisms of post-traumatic hyperinflammation and sepsis. *Immun Infekt* 1994; 22:203-13
26. Mynster T, Dybkjaer E, Kronborg G, Nielsen HJ: Immunomodulating effect of blood transfusion: Is storage time important? *Vox Sang* 1998; 74:176-81
27. Heiss MM, Mempel W, Jauch KW, Delanoff C, Mayer G, Mempel M, Eissner HJ, Schildberg FW: Beneficial effect of autologous blood transfusion on infectious complications after colorectal cancer surgery. *Lancet* 1993; 342:1328-33
28. Avall A, Hyllner M, Bengtson JP, Carlsson L, Bengtsson A: Postoperative inflammatory response after autologous and allogeneic transfusion. *ANESTHESIOLOGY* 1997; 87:511-6
29. Blumberg N: Allogeneic transfusion and infection: Economic and clinical implications. *Semin Hematol* 1997; 34S2:34-40
30. Heiss MM, Mempel W, Delanoff C, Jauch KW, Gabka C, Mempel M, Dieterich HJ, Eissner HJ, Schildberg FW: Blood transfusion-modulated tumor recurrence: First results of a randomized study of autologous versus allogeneic blood transfusion in colorectal cancer surgery. *J Clin Oncol* 1994; 12:1859-67
31. Innerhofer P, Walleczek C, Luz P, Hobisch-Hagen P, Benzer A, Stöckl B, Hessenberger G, Nussbaumer W, Schobersberger W: Transfusion of buffy coat-depleted blood components and risk of postoperative infection in orthopedic patients. *Transfusion* 1999; 39:625-32
32. Pertilä JT, Salo MS, Jalonen JR, Kuttala KT, Viinämäki O, Pulkki KJ: Blood transfusion with autologous and leucocyte-depleted or standard allogeneic red blood cells and the immune response to open heart surgery. *Anesth Analg* 1994; 79:654-60
33. Tietze M, Kluter H, Troch M, Kirchner H: Immune responsiveness in orthopedic surgery patients after transfusion of autologous or allogeneic blood. *Transfusion* 1995; 35:378-83
34. Jensen LS: Clinical importance of leukocyte depletion in surgical patients. *Infusionsther Transfusionsmed* 1998; 25:288-94
35. Mincheff MS, Meryman HT: Blood transfusion, blood storage and immunomodulation. *Immunol Invest* 1995; 24:303-9
36. Morikawa Y, Furotani M, Matsuura N, Kakudo K: The role of antigen-presenting cells in the regulation of delayed-type hypersensitivity. II. Epidermal Langerhans' cells and peritoneal exudate macrophages. *Cell Immunol* 1993; 152:200-10
37. Fredens K, Dybdahl H, Dahl R, Baandrup U: Extracellular deposit of the cationic proteins ECP and EPX in tissue infiltrations of eosinophils related to tissue damage. *APMIS* 1988; 96:711-9
38. Nielsen HJ, Reimert CM, Pedersen AN, Brünner N, Edvardsen L, Dybkjaer E, Kehlet H, Skov PS: Time-dependent, spontaneous release of white cell- and platelet-derived bioactive substances from stored human blood. *Transfusion* 1996; 36:960-5
39. Peterson CBG, Skoog V, Venge P: Human eosinophil cationic proteins (ECP & EPX) and their suppressive effect on lymphocyte proliferation. *Immunology* 1986; 171:1-13
40. Ness PM, Walsh PC, Zahurak M, Baldwin ML, Piantadosi S: Prostate cancer recurrence in radical surgery patients receiving autologous or homologous blood. *Transfusion* 1992; 32:31-6
41. Mynster T, Hammer JH, Nielsen HJ: Prestorage and bedside leucofiltration of whole blood modulates storage-time-dependent suppression of in vitro TNF α release. *Br J Haematol* 1999; 106:248-51
42. Mynster T, Nielsen HJ: Prestorage leucofiltration of red blood cell suspensions modulates storage time-dependent suppression of in vitro cytokine release. *Infus Ther Transfus Med* 2000; 27:244-9
43. Feola M, Simoni J, Tran R, Lox CD, Canizaro PC: Toxic factors in the red blood cell membrane. *J Trauma* 1989; 29:1065-75
44. Grover GJ, Loegering DJ: Effect of splenic sequestration of erythrocytes on splenic clearance function and susceptibility to septic peritonitis. *Infect Immun* 1982; 36:96-102