Background: Allogeneic or autologous blood seems to have an immunosuppressive effect that is largely attributable to storage-dependent factors. However, transfusion of postoperative unwashed shed blood (USB) after elective total knee replacement does not undergo storage. Therefore, the authors explored the effects of USB on the mitogen-driven cytokine synthesis by the patient’s peripheral blood mononuclear cells.

Methods: Perioperative blood samples were obtained from 12 total knee replacement patients with and 5 without reinfusion of leukoreduced USB, and from USB reinfusion line, before and after leukoreduction. Venous blood obtained at 4–6 postoperative hours was coincubated with USB. Endotoxin-stimulated release of tumor necrosis factor α and interleukin 10 was measured after 24 h of culture by solid-phase enzyme-labeled chemiluminescent immunometric assay.

Results: Coincubation of postoperative venous blood with USB, USB cells, or USB plasma resulted in a significant depression of tumor necrosis factor-α synthesis, without significant influences on interleukin-10 synthesis. However, no differences were observed for endotoxin-stimulated cytokine release in perioperative blood samples from patients receiving or not receiving USB.

Conclusion: These data suggest that USB seemed to contain an antiinflammatory agent. However, at the actual retransfusion rate, USB does not seem to further enhance the immunosuppression that follows knee replacement surgery.

It is well known that general and local infection continues to be one of the main causes of morbidity and mortality associated with orthopedic and trauma surgery, with a higher incidence in patients receiving allogeneic blood transfusions (ABTs). Unilateral total knee replacement (TKR) can result in a substantial blood loss, and 20–50% of these patients receive ABTs, which may increase the risk of postoperative infection. In addition, allogeneic blood is not risk free, and different methods to reduce or avoid ABTs in these patients have been developed.

In this regard, transfusion of postoperative unwashed shed blood (USB) has become a popular blood saving strategy after major orthopedic procedures. Despite its effectiveness, several authors have questioned the safety of this blood-saving method because USB may contain activated leukocytes or inflammatory mediators, which might be harmful for the patient. However, reinfusion of USB, with or without leukoreduction, after TKR does not modify acute phase response induced by surgery. Moreover, return of whole USB seems to activate systemic immunity after joint replacement as shown by increased frequencies of natural killer cell precursors and synthesis of interferon-γ, and by increased production of reactive oxygen species by neutrophils.

However, it was found that banked autologous whole blood has an immunosuppressive effect that is largely attributable to storage-dependent factors. Unlike preoperative autologous blood donation, USB does not undergo storage, and this may result in significant differences with respect to its immunomodulatory effects. In this study, we explored the effects of USB on the ability of leukocytes cultured in an in vitro model of blood transfusion to release the prototypical proinflammatory and antiinflammatory cytokines, tumor necrosis factor α (TNF-α) and interleukin 10 (IL-10).

Materials and Methods

Patients and Surgical Procedure

After approval by the institutional review board (University Hospital Virgen de la Victoria, Málaga, Spain), 20 consecutive patients undergoing surgery for TKR who gave informed consent entered the study. Patients with hematologic diseases or coagulation disorders, patients with hepatic or renal diseases, those under antiinflammatory or immunosuppressive therapy, having surgery or transfusion in the previous 3 months, or with known infection or malignancy at admission were excluded.

All surgical procedures were performed under intraarticular anesthesia (0.5% hyperbaric bupivacaine, 15 mg) and involved the application of a tourniquet that was deflated before knee closure and the use of a postoperative blood collection device. All patients received a total condylar knee (Duracon; Stryker, Kalamazoo, MI), gentamycin cement being used for the tibial component. Only saline solution (0.9% NaCl) was used for wound washing.

The anesthesiologist estimated fluid administration (1,000–1,500 ml lactated Ringer’s solution) and blood losses, both at the operation theater and in the anesthe-
sia recovery unit, and performed the reinfusion of USB. In the ward, measurement of postoperative blood loss and decisions on postoperative transfusions were made by the attending surgeon.

A set of demographic and clinical data was collected from all patients, including age, sex, tourniquet use and operation time, intraoperative and postoperative blood loss, volume and units of USB returned, perioperative hematocrit and hemoglobin, leukocyte and platelet counts, postoperative complications, and duration of hospital stay.

**Postoperative Blood Salvage and Reinfusion**

At the end of surgery, the collection blood canister (ConstaVac CBC II; Stryker) was connected to two drainage catheters through a Y-connector, and USB was collected without anticoagulant, at a negative pressure of 25 mmHg. The canister was connected to the reinfusion bag to which USB was transferred. The last 60–80 ml of the USB was discarded to minimize fat particles and prevent other debris substances being transfused to the patient. If at least 400 ml of blood was collected within the first 6 postoperative hours, return of USB was performed through a leukocyte-depletion filter (PureCell; Pall Biomedical, Portsmouth, United Kingdom) inserted into the patient’s line. The volume of recovered USB was converted into blood units according to the expression: $U = \text{USB volume (ml)} \times \text{USB hematocrit (％)} / 400 \text{ (ml)}$.

**Perioperative Blood Samples**

Several blood samples were collected: five from the patient (samples 1, 2, 5, 6, and 7) and two from the USB (samples 3 and 4). Patient’s samples were obtained before anesthesia; at the sixth postoperative hour, before reinfusion of USB; and on the first, third, and seventh postoperative days. USB samples were taken from the bag before reinfusion and from the reinfusion line after leukofiltration. Patient samples were collected into heparin plastic tubes (BD Vacutainer LH; Becton Dickinson, Plymouth, United Kingdom). All blood samples were obtained immediately before the subsequent in vitro investigation. Blood cell counts were determined in an automated analyzer (Pentra 120 Retic; ABX, Montpellier, France), and plasma aliquots were frozen at $-80^\circ \text{C}$ until they were assayed for cytokines.

**Ex Vivo Endotoxin Stimulation of Blood Samples and Cocultures**

The immunomodulatory effects of the various blood components were studied in a whole blood culture system, previously used to explore immunomodulatory effects of stored allogeneic and autologous blood transfusion. Briefly, USB blood (samples 3 or 4) was added to postoperative venous whole blood from the same patient (sample 2) and subjected to coculture (volume: volume ratio, 1:1) in the presence of a mitogenic endotoxin (lipopolysaccharide). To further characterize the possible contribution of cellular components as opposed to soluble factors present in postoperative whole USB (sample 3) or leukoreduced USB (sample 4), in an additional series of experiments, USB was centrifuged at 3,500g for 15 min, and the supernatant and cellular fractions were separately coincubated with postoperative venous whole blood from the same patient (sample 2) in a ratio of 1:1 (volume:volume).

Aliquots (2 ml) of each perioperative blood sample and blood coculture under study were stimulated by addition of 1 μg/ml lipopolysaccharide (Escherichia coli O111: B4; Sigma, St. Louis, MO) in a 24-well plate in a humidified atmosphere with 5% carbon dioxide at 37°C for 24 h. At 24 h after onset of culture, the supernatant was collected from the wells and stored immediately at $-80^\circ \text{C}$ until it was assayed for cytokines.

**Measurement of Cytokines in Plasma and Culture Supernatants**

Samples of perioperative blood plasma as well as samples of culture supernatants were assessed in duplicate for TNF-α and IL-10 concentrations by means of an automated solid-phase enzyme-labeled chemiluminescent immunometric assay (Immulite I; Diagnostics Products Corporation, Los Angeles, CA). Controls for the cytokines studied were provided by the manufacturer and measured in duplicate with each assay. Calculated intraassay and interassay coefficients of variance for controls and samples were less than 10% for both cytokines. Assay detection limits were 4 pg/ml for TNF-α and 5 pg/ml for IL-10. Samples were thawed at room temperature and were diluted 1:10 to 1:20 with plasma obtained from healthy donors to stay within the linear range of the assay.

For comparison with preoperative values, the patient’s postoperative blood culture supernatant TNF-α and IL-10 concentrations were corrected for changes in mononuclear leukocyte counts and plasma volume, according to the expression

$$\text{Cytokine}_{\text{corrected}} = \frac{\text{Cytokine}_{\text{measured}} \times (\text{PBMC}_{\text{pre}}/\text{PBMC}_{\text{post}}) \times (\text{PV}_{\text{post}}/\text{PV}_{\text{pre}})}{}$$

where PBMC = peripheral blood mononuclear leukocytes, PV = plasma volume, pre = preoperative blood, and post = postoperative blood.

For whole blood coculture assays, the anticipated value for lipopolysaccharide-stimulated TNF-α and IL-10 release (expected value) was assumed to be the arithmetic mean of the value obtained for postoperative blood (sample 2) and whole USB samples (samples 3 or 4) after stimulation with lipopolysaccharide. For calculation of expected values in the coculture assay with USB, the contribution of USB was corrected for increase...
in cytokine-producing cells and for reduction in plasma volume, according to the expression

\[
\text{Cytokine}_{\text{expected}} = \frac{(\text{Cytokine}_{\text{post}} \times PV_{\text{post}}) + (\text{Cytokine}_{\text{USB}} \times PV_{\text{USB}} \times \text{factor})}{(PV_{\text{post}} + PV_{\text{USB}})},
\]

where \(PV\) = plasma volume, \(post\) = postoperative blood, \(USB\) = unwashed shed blood, \(USBc\) = unshed blood cells, and \(factor = \frac{Htc_{\text{USBc}}}{Htc_{\text{USB}}}\).

For calculation of expected value in coculture assay using \(USB\) supernatants, lipopolysaccharide-stimulated cytokine release was corrected for the dilution produced by the addition of USB plasma, according to the expression

\[
\text{Cytokine}_{\text{expected}} = \frac{(\text{Cytokine}_{\text{post}} \times PV_{\text{post}})}{(PV_{\text{post}} + 1)},
\]

where \(PV\) = plasma volume, and \(post\) = postoperative blood.

Statistical Analysis
Data are reported as mean \(\pm\) SD or as median and range. Statistical differences were determined by the nonparametric Wilcoxon rank test for culture and coculture data. Demographic and clinical variables were compared with the parametric Student \(t\) test or nonparametric Pearson chi-square test. For repeated measures, statistical analysis was conducted using a multivariate analysis of variance test with a within-factor (up to four levels) and one between-factor (group). Statistical tests were performed using the SPSS 12.0 package (SPSS Inc., Chicago, IL), licensed to the University of Málaga, Málaga, Spain. All \(P\) values reported are two-sided and are considered statistically significant at less than 0.05.

Results
Demographic Data and Clinical Outcomes
Twenty consecutive patients (15 women and 5 men) underwent surgery for TKR. Twelve patients were reinfused with \(USB\), and 8 were not reinfused with \(USB\). \(USB\) patients had a mean postoperative blood loss of 743 ml, with 552 ml (equivalent to 1 unit of packed erythrocytes) being returned per patient, without any clinically relevant incident. No patient received \(ABT\)s or had development of postoperative complications. Three of 8 patients not receiving \(USB\) were excluded because of \(ABT\)s (1), urinary tract infection (1), and inadequate venous access (1), and the remaining 5 patients were the control group. There were no differences in sex distribution, weight, height, operation time, tourniquet time, or mean duration of hospital stay between groups. The 48-h blood loss was higher in the \(USB\) group than in the control group (table 1). None of the patients had abnormal preoperative platelet or leukocyte counts, and there were no differences between groups in perioperative hemoglobin levels or perioperative leukocyte and platelet counts (table 2), but 2 men in the \(USB\) group had anemia (hemoglobin < 13 g/dl). Plasma concentrations of TNF-\(\alpha\) and IL-10 were low or undetectable in preoperative and postoperative venous blood and moderate in postoperatively salvaged unwashed shed blood (table 2).

Lipopolysaccharide-stimulated Cytokine Release Assay
At 24 h after onset of culture in the presence of lipopolysaccharide, TNF-\(\alpha\) and IL-10 concentrations in supernatants of lipopolysaccharide-stimulated USB, with or without leukodepletion, were significantly lower than those observed in lipopolysaccharide-stimulated preoperative venous blood (table 3). In the control group, lipopolysaccharide-stimulated TNF-\(\alpha\) secretion by samples taken at 4–6, 24, and 72 h after surgery were lower than that of preoperative sample, whereas in the USB group, the difference was only significant between lipopolysaccharide-stimulated TNF-\(\alpha\) secretion by preoperative and 4- to 6-h postoperative samples (table 3). Lipopolysaccharide-stimulated IL-10 secretion by all postoperative blood samples was significantly lower than that of preoperative samples in both groups (table 3). However, no differences in the postoperative time course of lipopolysaccharide-stimulated TNF-\(\alpha\) and IL-10 secretion were observed between groups (multivariate analysis of variance test; table 3).

Effect of the Different Blood Components on Lipopolysaccharide-stimulated TNF-\(\alpha\) and IL-10 Release
Coincubation of postoperative venous blood with equal amounts of \(USB\), with or without leukoreduction, reduced lipopolysaccharide-stimulated TNF-\(\alpha\) secretion below the expected values (\(P < 0.05\)), but it had no significant effect on the lipopolysaccharide-induced re-
Interleukin 10, pg/ml

Leukocytes, x10^9/μl

Platelets, x10^9/μl

Table 3. Cytokine Concentrations in Perioperative Blood Samples after Ex Vivo Cultured with Lipopolysaccharide

<table>
<thead>
<tr>
<th>Blood Sample</th>
<th>TNF-α, pg/ml Control (n = 5)</th>
<th>TNF-α, pg/ml USB (n = 12)</th>
<th>Interleukin 10, pg/ml Control (n = 5)</th>
<th>Interleukin 10, pg/ml USB (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preoperative</td>
<td>3,740 (2,680–9,740)</td>
<td>2,910 (2,080–10,000)</td>
<td>3,740 (897–6,450)</td>
<td>3,070* (1,610–6,880)</td>
</tr>
<tr>
<td>Postoperative 4–6 h</td>
<td>1,556* (501–3,205)</td>
<td>1,616* (412–3,070)</td>
<td>645* (208–4,856)</td>
<td>1,124* (323–6,885)</td>
</tr>
<tr>
<td>Postoperative day 1</td>
<td>2,057* (1,134–3,195)</td>
<td>3,224 (1,216–9,003)</td>
<td>52* (385–2,967)</td>
<td>1,381* (252–2,951)</td>
</tr>
<tr>
<td>Postoperative day 3</td>
<td>2,909* (987–3,400)</td>
<td>2,784 (1,918–10,681)</td>
<td>772* (608–1,558)</td>
<td>1,158* (156–1,599)</td>
</tr>
<tr>
<td>Postoperative day 7</td>
<td>3,437 (2,178–6,616)</td>
<td>4,139 (1,568–14,086)</td>
<td>1,052* (561–3,019)</td>
<td>1,536* (565–3,002)</td>
</tr>
<tr>
<td>Whole shed blood</td>
<td>—</td>
<td>466* (258–1,670)</td>
<td>—</td>
<td>273* (70–790)</td>
</tr>
<tr>
<td>Leukoreduced shed blood</td>
<td>—</td>
<td>177* (146–368)</td>
<td>—</td>
<td>65* (21–156)</td>
</tr>
</tbody>
</table>

Data are presented as median (range) of n duplicate measurements. Samples were taken from patients undergoing total knee replacement receiving postoperative unwashed shed blood (USB; n = 12) or no transfusion (control; n = 5).

* P < 0.05, preoperative vs. shed blood samples.

TNF-α = tumor necrosis factor α.
cytokine response to unwashed blood autotransfusion

Table 4. Effects of Various Blood Products Obtained from USB on Lipopolysaccharide-stimulated Response of Cultured Postoperative Patient’s Whole Blood

<table>
<thead>
<tr>
<th>Coculture</th>
<th>n</th>
<th>Expected (range)</th>
<th>Observed (range)</th>
<th>Expected (range)</th>
<th>Observed (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postoperative blood + whole USB</td>
<td>12</td>
<td>1,003 (620–2,900)</td>
<td>621* (388–933)</td>
<td>1,095 (178–2,687)</td>
<td>1,340 (637–2,670)</td>
</tr>
<tr>
<td>Postoperative blood + leukoreduced USB</td>
<td>12</td>
<td>875 (242–2,140)</td>
<td>388* (280–873)</td>
<td>750 (130–2,510)</td>
<td>1,400 (355–2,500)</td>
</tr>
<tr>
<td>Postoperative blood + whole USB cells</td>
<td>12</td>
<td>2,157 (1,478–6,318)</td>
<td>708* (326–1,630)</td>
<td>2,811 (536–4,994)</td>
<td>3,015 (609–4,870)</td>
</tr>
<tr>
<td>Postoperative blood + leukoreduced USB cells</td>
<td>12</td>
<td>1,958 (1,131–3,553)</td>
<td>726* (301–1,570)</td>
<td>1,639 (294–4,194)</td>
<td>3,005 (895–5,810)</td>
</tr>
<tr>
<td>Postoperative blood + whole USB plasma</td>
<td>12</td>
<td>770 (286–1,642)</td>
<td>459* (322–920)</td>
<td>685 (101–1,958)</td>
<td>707 (271–1,650)</td>
</tr>
<tr>
<td>Postoperative blood + leukoreduced USB plasma</td>
<td>12</td>
<td>772 (367–1,448)</td>
<td>425* (246–848)</td>
<td>650 (98–1,934)</td>
<td>614 (117–1,550)</td>
</tr>
</tbody>
</table>

Data are presented as median (range) of n duplicated determinations. The patient’s postoperative blood sample was taken at postoperative hours 4–6. Values were corrected for dilution and/or for change in cytokine producing cells, as stated in the Materials and Methods.

* P < 0.05 (Wilcoxon rank test).

TNF-α = tumor necrosis factor α; USB = unwashed shed blood.

after joint replacement as shown by increased frequencies of natural killer cell precursors and concentration of interferon γ. However, it must be considered that in the above-mentioned study, USB was collected in acid citrate dextrose, and the blood salvage protocol allowed for several USB returns, with up to 1,500 ml nonleukoreduced USB being given back to patients, whereas in our study, USB was collected without anticoagulant, a relatively small volume of leukoreduced USB was returned once (approximately 500 ml), and none of the studied patients received ABTs or preoperatively donated autologous blood.

In our in vitro model, patient’s venous blood drawn at postoperative hours 4–6 served as “recipient,” cocultures of venous blood with USB simulated the reinfusion process, and endotoxin stimulation simulated the response to infection. In the presence of USB, we found that TNF-α release, but not that of IL-10, was reduced, and these effects were not eliminated by leukofiltration. The magnitude of the reduction of TNF-α secretion was similar to that observed by Biedler et al., for cocultures of fresh venous blood with stored allogeneic or autologous blood. However, in contrast to our findings, an increase in IL-10 secretion was observed in this later experimental setting (110% and 142%, respectively). Moreover, the overall effects of USB on lipopolysaccharide-stimulated cytokine release were lower than that observed for either nonleukoreduced allogeneic or autologous stored blood but almost identical to that produced by allogeneic leukoreduced erythrocytes.

To further address the relative role of humoral as opposed to cellular factors mediating the effects of USB, additional experiments were conducted in which plasma and cellular fractions of whole and leukoreduced USB were separated by centrifugation and incubated selectively with postoperative venous blood. These experiments did not reveal a discriminate role for plasma and cellular components in mediating the effects of USB. For whole and leukoreduced USB, the inhibitory effect of on TNF-α was mediated by both plasma and cellular fractions.

However, it must be noted that, according to the experimental design, plasma:blood cell ratio varies for the different coincubation assays. When USB supernatant was coincubated with postoperative venous blood, we used 1.5 times as much supernatant as in the assays with whole USB. Substances known to be released into USB from the knee joint site include IL-1β, IL-6, IL-8, and IL-10, complement degradation products (C-3a, C-5b, SC-5b-9), and constituents of methyl methacrylate cement such as gentamycin, microparticles of polyethylene, and some metals (cobalt, chromium, molybdenum, nickel), making it difficult to know which are responsible for depression of TNF-α release. However, IL-6, an antiinflammatory cytokine that is a potent inhibitor of TNF-α release, seems to be a suitable candidate because its concentration in USB plasma is very high and not removable by filtration. This is in agreement with the observation by Biedler et al. that plasma factors released into stored blood are mainly responsible for the depression of TNF-α release in this model.

Similarly, when USB cells were coincubated with postoperative venous blood, we used 3 times as many shed blood cells as in the assays with whole USB. The mechanisms by which USB cells reduced endotoxin-induced TNF-α release is unclear, because it was also observed after coincubation with leukoreduced whole USB and USB cells, suggesting that this effect is not induced by leukocytes and other blood components may be involved. In this respect, USB presented a higher degree of hemolysis than stored blood, as shown by the increased concentration of plasma free hemoglobin. Lysed erythrocytes might play a role, because erythrocyte membrane phospholipids were shown to activate macrophage-derived prostaglandins, which are potent immune regulatory factors. In addition, platelets are present in USB, although in low concentrations, and they may have been activated by extravasation, contact with the blood salvage device and tubing, and filtration. Activated plate-
lets may modulate leukocyte activity by the release of several cytokines, such as transforming growth factor β. Transforming growth factor β is a family of proteins with diverse effects, including suppression of inflammation and, possibly, transfusion-related immunomodulation.

Finally, the effects of the remaining plasma in the USB cell concentrate (15%) may also contribute to depression of TNF-α release. In contrast to our findings, Biedler et al. showed that the cellular component of stored allogeneic whole blood exerts a stimulatory effect on lipopolysaccharide-induced TNF-α release when added to fresh venous blood in the coculture system.

As expected from the results of experiments with whole USB, lipopolysaccharide-induced IL-10 release was not significantly affected by coincubation with either the plasma or the cellular fraction of whole or leukoreduced USB (table 4). This is in contrast to the observation by Biedler et al. that cellular components are mainly responsible for the increased release of IL-10 after addition of stored blood to the coincubation system.

Finally, given that (1) the transfusion ratio used in the experimental study (1:1) far exceeded that used in the clinical setting for both USB and ABT (1:10 to 1:5; transfused volume:patient’s blood volume), (2) the effects on lipopolysaccharide-stimulated cytokine secretion of the transfused components in the in vitro model of transfusion seem to be type- and dose-dependent, being similar for USB and allogeneic leukoreduced packed erythrocytes, when added to the same volume of recipient blood; and (3) we found no significant effect of USB reinfusion on the patient’s blood response to lipopolysaccharide, it would also be conceivable that the transfusion of 1–2 units of leukoreduced allogeneic erythrocyte concentrate (which is currently the standard hemotherapy in our hospitals) or washed shed blood to patients undergoing major orthopedic surgery neither enhances the immunosuppressive effect associated with TKR nor increases the risk of postoperative infection. To gain more insight into the effects of USB on postoperative immune status, additional studies with different transfusion therapies are essential.

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References


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