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Blood Transfusion Promotes Cancer Progression: A Critical Role for Aged Erythrocytes

Shir Atzil, M.A., Michal Arad, M.A.,* Ariella Glasner, M.A.,* Noa Abiri, M.A.,* Roi Avraham, M.A.,* Keren Greenfeld, M.A.,* Ella Rosenne, M.A.,* Benzion Beilin, M.D.,† Shamgar Ben-Eliyahu, Ph.D.‡*

Background: **In cancer patients, allogeneic blood transfusion is associated with poorer prognosis, but the independent effect of the transfusion is controversial. Moreover, mediating mechanisms underlying the alleged cancer-promoting effects of blood transfusion are unknown, including the involvement of donors' leukocytes, erythrocytes, and soluble factors.**

Method: **Two syngeneic tumor models were used in Fischer 344 rats, the MADB106 mammary adenocarcinoma and the CRNK-16 leukemia. Outcomes included host ability to clear circulating cancer cells, and host survival rates. The independent impact of blood transfusion was assessed, and potential deleterious characteristics of the transfusion were studied, including blood storage duration; the role of erythrocytes, leukocyte, and soluble factors; and the kinetics of the effects.**

Results: **Blood transfusion was found to be an independent and significant risk factor for cancer progression in both models, causing up to a fourfold increase in lung tumor retention and doubling mortality rates. Blood storage time was the critical determinant of these deleterious effects, regardless of whether the transfused blood was allogeneic or autogenic. Surprisingly, aged erythrocytes (9 days and older), rather than leukocytes or soluble factors, mediated the effects, which occurred in both operated and nonoperated animals. The effects of erythrocytes transfusion in the MADB106 model emerged immediately and dissipated within 24 h.**

Conclusions: **In rats, transfusion of fresh blood is less harmful than transfusion of stored blood in the context of progressing malignancies. Further studies should address mediating mechanisms through which erythrocytes' storage duration can impact the rate of complications while treating malignant diseases and potentially other pathologies.**

THROUGHOUT the history of medicine, the beneficial outcomes of allogeneic blood transfusion have been coupled with adverse reactions, including host responses to incompatible erythrocyte determinants, infections, and transfusion-associated immune modulations.[1,2] Notably, patients receiving blood transfusion before organ transplantation have long been reported to exhibit improved graft and patient survival.[3,4] With additional research, it became evident that allogeneic leukocytes present in the transfused blood often suppress host cellular immune responses, particularly those mediated by T lymphocytes and natural killer (NK) cells.[5,6] This suppression is believed to underlie reduced host-antigraft responses[7] and increased patients susceptibility to infections.[1,2,5,6]

Cancer progression was also suggested to be affected by blood transfusion. Animal studies conducted by Blajchman *et al.* elegantly indicated a cancer-promoting effect of allogeneic blood transfusion, using various animal models.[8–10] These studies also suggested the involvement of recipients' cellular immune mechanisms in mediating these effects. In humans, the majority of retrospective and some prospective studies have indicated poorer prognosis in cancer patients receiving allogeneic blood transfusion during the perioperative period. These findings were reported in patients harboring gastric, colorectal, lung, head, neck, prostate, and breast cancers.[1,11] This suggests the generalizability of the findings, irrespective of cancer type or specific hospital routine.

However, given ethical considerations in human studies, it cannot be determined whether blood transfusion *per se*, during the perioperative period, is an independent risk factor for postoperative cancer recurrence. It is feasible that the circumstances necessitating blood transfusion, rather than the transfusion itself, underlie such outcomes.[1,7,11,12] Randomized clinical trials that were performed compared different regimens of transfusion, including leukocyte depletion *versus* nondepletion. Therefore, these trials could not have assessed the role of blood constituents that cannot be removed from the transfusion, specifically erythrocytes, and could not have indicated the independent impact of the transfusion as a whole.

An additional unresolved issue is the potential role of donors' leukocytes in mediating cancer progression in transfused patients. Whereas allogeneic leukocytes were implicated in mediating various adverse effects of blood transfusion, there is no conclusive evidence that they can worsen cancer progression.[1] A large randomized clinical trial comparing leukocyte-containing regimens with leukocyte-depleted regimens concluded that allogeneic leukocytes have no effect on colorectal cancer recurrence, because both conditions showed similar de-

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* Graduate Student, Neuroimmunology Research Unit, Department of Psychology, Tel Aviv University. † Associate Professor and Head, Department of Anesthesiology, Rabin Medical Center, Golda-Hasharon Campus, Sackler School of Medicine, Tel Aviv University. ‡ Professor and Head, Neuroimmunology Research Unit, Department of Psychology, Tel Aviv University; OUTCOMES RESEARCH Institute, Louisville, Kentucky.

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Address correspondence to Dr. Ben-Eliyahu: Department of Psychology, Tel Aviv University, Tel Aviv, 69978, Israel. shamgar@post.tau.ac.il.

cline in survival rates compared with nontransfused patients.[13] Other studies have similarly reported no beneficial effects of leukodepletion in cancer patients.[14]

In the current study, we aimed at addressing some of the unavoidable shortcomings of human studies. Specifically, we (1) tested whether blood transfusion is an independent risk factor for cancer progression; (2) studied the impact of indispensable blood constituents, including erythrocytes; and (3) studied the impact of blood storage duration in allogenic and autogenic transfusion. To this end, we used two nonimmunogenic tumor lines syngeneic to the F344 rat used herein: the MADB106 mammary adenocarcinoma and the CRNK-16 leukemia. We tested cancer progression by monitoring rats' ability to clear circulating cancer cells and by assessing survival rates. The impact of blood transfusion in the context of surgery was also studied.

Materials and Methods

Animals and Counterbalancing

F344 rats (females or males) were purchased from Harlan Laboratories (Jerusalem, Israel), and housed four per cage under a 12-h light/12-h dark cycle with free access to food and water. Animals were acclimatized to the vivarium for at least 4 weeks before experimentation and were aged 12–20 weeks at that time. In any given experiment, all animals were of the same age and sex. To minimize experimental stress, all rats were handled daily for 3 days before each study. The order of blood transfusion and tumor injection was counterbalanced across groups in each experiment. Control animals were transfused with saline or syngeneic fresh blood. Donors were 6-month-old male Wistar (allogenic) or F344 rats (syngeneic/autogenic) housed under the same conditions. The characteristics of the donors (age, sex, *etc.*) were counterbalanced across recipient groups. All studies were approved by The Institutional Animal Care and Use Committee of Tel Aviv University (Tel-Aviv, Israel) and performed in accordance with relevant guidelines and regulations.

Blood Collection, Preparation of Blood Constituencies for Transfusion, and Storage

Donor rats were overdosed with halothane, and approximately 18 ml blood from each Wistar rat (allogenic blood) or 11 ml blood from each inbred F344 rat (syngeneic/autogenic blood) was drawn from the heart into syringes containing citrate–phosphate dextrose solution with adenine (Sigma, Rehovot, Israel; 1:7 citrate:blood vol/vol). For preparation of packed cells, blood was centrifuged for 20 min at 850*g*, the supernatant was disposed of, and the remaining packed cells were stored at 4°C. For leukoreduction, the serum fraction and the leukocyte buffy coat were removed after centrifugation by manual pipette aspiration. Remaining leukocytes were then counted in each sample before transfusion. To separate leukocytes from erythrocytes before storage, we first used a modified Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) separation procedure. Specifically, 10 ml blood was diluted with equal volume of saline and placed on 12 ml Ficoll-Paque solution in 50-ml tubes. Samples were centrifuged for 10 min at 750 *g*, and the layers above the erythrocytes, which contained most of the leukocytes, were collected. Leukocytes remaining on top of the erythrocyte fraction were removed manually by a pipette, which also removed approximately 20% of the erythrocyte volume that was discarded. Leukocytes and erythrocytes were then washed three times in 40 ml phosphate-buffered saline and stored as packed cells. All procedures were conducted under sterile conditions. Unlike standard Ficoll-Paque separation procedures, this procedure enables collecting most of the leukocytes, including granulocytes. Fluorescence-activated cell-sorting analysis and hemocytometric assessment indicated that the harvested leukocytes contained 70–90% of the original leukocyte number, including all leukocyte subpopulations. The erythrocyte fraction contained less than 10% of the original leukocyte number, including all leukocyte subpopulations. For leukodepletion—to thoroughly remove leukocytes from the blood—we used high-efficiency leukocyte removal filters (Pall Purecell NR; Pall, Portsmouth, United Kingdom), which are routinely used clinically and known to reduce leukocytes by a factor greater than 10^5. Using these filters, we routinely inspected for remaining leukocytes and were able to verify that at least 99.99% of leukocytes were removed.

Transfusion of Blood Constituencies, and Tumor Inoculation

Immediately before transfusion, blood constituents (*i.e.*, packed cells, only erythrocytes, only leukocytes, only supernatant from stored packed cells) were filtered through a 40-μm membrane, to remove or break up potential aggregates. F344 rats were anesthetized with halothane (vaporizer, 2.5%), and a 24-gauge intravenous cannula was inserted into the tail vein. A standard 3-ml volume containing different quantities and fractions of saline-diluted blood constituents was slowly transfused during a 10-min period. Three milliliters saline containing the same quantities of citrate–phosphate dextrose solution with adenine as in the transfused constituent was used for control transfusion. Unless otherwise indicated, the transfusion content originated from 3 ml of the donor's blood. Tumor cells were inoculated at the end of this 10-min period through the intravenous cannula (except in the first experiment, in which tumor was given at different time points).

Tumor Cell Lines and Tumor Models

MADB106 Tumor Line. MADB106 is a selected variant cell line obtained from a pulmonary metastasis of a mammary adenocarcinoma chemically induced in the inbred F344 rat.[15] The MADB106 tumor metastasizes only to the lungs after its intravenous inoculation, and is known to be sensitive to NK cell activity *in vivo*.[15–19] After intravenous inoculation of MADB106 tumor cells, metastases are formed only in the lungs, and lung tumor retention of MADB106 cells is an early indicator of this outcome.[15,17,18] Cell were maintained at 5% CO_2, 37°C, 100% humidity, in monolayer cultures in complete medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 50 μg/ml gentamicin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate). Cells were separated from the flask using 0.25% trypsin.

Radiolabeling of MADB106 Cells and Assessment of Lung Tumor Retention. DNA radiolabeling of tumor cells was accomplished by adding 0.4 μCi/ml ^{125}Iododeoxyuridine to the growing cell culture 1 day before harvesting the cells. Labeled MADB106 tumor cells, 4×10^5/kg, in 0.5 ml phosphate-buffered saline were injected into the tail vein during halothane anesthesia. Tumor cells were always administered immediately after transfusion (excluding the first study). Twenty-one hours after tumor inoculation, rats were euthanized with halothane, and their lungs were removed and placed in a gamma counter for assessment of radioactive content. The percentage of tumor retention was calculated as the ratio between radioactivity measured in the lungs and total radioactivity in the injected tumor cell suspension. Our previous studies have indicated that the levels of lung radioactivity reflect the numbers of viable tumor cells in the lungs.[17]

CRNK-16 Tumor Cells. The CRNK-16 cell line is derived from a naturally occurring leukemia that is highly malignant and is a major cause of natural death in aged F344 rats.[20] The CRNK-16 cell line was maintained in complete medium at 100% humidity, 5% CO_2 at 37°C.

Inoculation of CRNK-16 Cells and Assessment of Survival. During halothane anesthesia, 60 CRNK-16 cells were injected into a rat tail vein in 0.5 ml phosphate-buffered saline immediately after blood transfusion. Beginning a week after tumor inoculation, rats were inspected daily for morbidity. Specifically, we euthanized rats that became indifferent or irresponsive to environmental stimuli, that showed motor difficulties, or that lost more than 10% of their body weight. We inspected euthanized rats for the development of solid tumors in internal organs (specifically spleen, liver, kidney, and all organs in the chest cavity) or spinal cord, and identified a malignant development in all morbid rats. Based on our experience with this tumor model,[19] these malignancies characterize a progressive stage of this cancer, causing death in the approximating 24–48 h. Therefore, these animals are included in the mortality report and were considered to have died on the next day. No morbidity was detected past day 82, and rats were inspected until day 112. None of the survivors showed any signs of illness throughout the study.

Surgical Procedure (Laparotomy)

Anesthesia was induced with halothane and maintained at a concentration of 2–3%. After hair trimming and scrubbing with alcohol, a 4-cm midline abdominal incision was performed, and the small and large intestines were externalized and covered with phosphate-buffered saline–soaked gauze. Before closing, the intestines were repositioned, and the skin and muscle were closure in one layer with four or five sutures. The procedure requires 20 min to conduct.

Statistical Analysis

For the MADB106 experiments, analysis of variance was conducted, using percentage lung tumor retention as the dependent index. Provided that significant group differences existed, two-sided protected least significant difference contrasts for pairwise group comparisons were used. All data are presented as mean ± SEM. For the CRNK-16 survival study, the Kaplan–Meier model was used for survival analysis, followed by the pairwise two-tailed Tarone–Ware test of group comparisons. For all experiments, a P value of less than 0.05 was considered significant, and all P values were two-tailed. The StatView 5.0 (Cary, NC) statistical package was used for statistical analysis.

Results

Immediate and Short-lasting Effect of Blood Transfusion

To test whether and for how long blood transfusion can affect the clearance of MADB106 tumor from the lungs, allogenic blood stored as packed cells for 12 days (or saline for control) was transfused at 24, 4, 1, or 0 h before MADB106 tumor cell inoculation or 1 h after tumor inoculation. Blood transfusion significantly increased percentage lung tumor retention up to fivefold compared with saline transfusion ($P < 0.05$) when given at all time points (*e.g.*, from 0.055 ± 0.012 to 0.259 ± 0.044 at the 0-h time point; $P < 0.05$), except when given 24 h before the tumor, in which blood transfusion did not cause any elevation in lung tumor retention and no significant effect (fig. 1). This indicates an immediate and short-lasting deleterious effect of blood transfusion on this index of cancer progression.

Effect of Storage Interval and Histocompatibility

To test whether different storage intervals and histocompatibility influence the effect of blood transfusion,

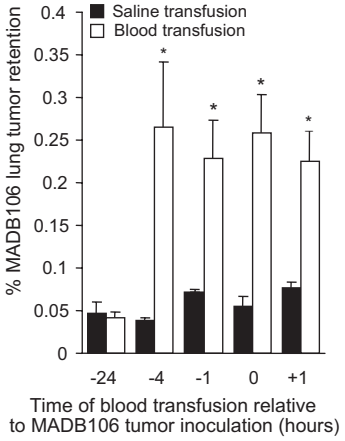

Fig. 1. Blood transfusion increases MADB106 lung tumor retention when transfused in close time proximity to tumor inoculation. Percentage of lung tumor retention (mean ± SEM) in rats transfused at several time points before MADB106 tumor inoculation (−24, −4, or −1 h), simultaneously with tumors (0), or 1 h after tumor inoculation (+1) compared with corresponding saline transfusion. n = 44; 3–6 per group. * Significant pairwise difference from the same time point saline control group (protected least significant difference, $P < 0.05$).

allogenic or autogenic blood was stored as packed cells for 0, 3, 9, 12, or 14 days before transfusion. Blood of either allogeneic or syngeneic origin stored for 9 days or longer significantly increased percentage lung tumor retention ($P < 0.05$) in a storage time–dependent manner compared with saline transfusion (*e.g.*, saline: 0.150 ± 0.015, 0-day storage: 0.120 ± 0.013, 14-day storage: 0.501 ± 0.082) in the allogeneic transfusion ($P < 0.05$; fig. 2). Fresh blood, allogeneic or syngeneic, had no deleterious effect.

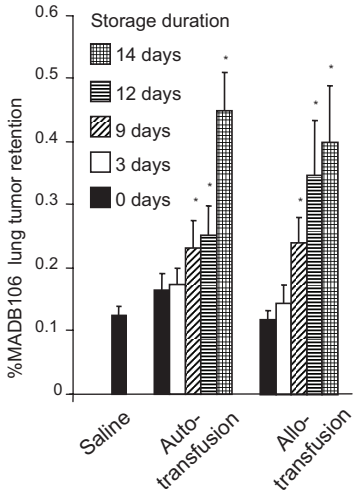

Fig. 2. Both allogeneic and autogenic blood transfusions increased lung tumor retention in a storage time–dependent manner. Percentage of lung tumor retention (mean ± SEM) in rats transfused with saline, allogeneic blood (allotransfusion), or autogenic blood (autotransfusion) that was stored for various durations as packed cells. * Significant pairwise difference from the saline control group (protected least significant difference, $P < 0.05$). n = 104; saline and 9 days, 12–14 per group; other groups, 4–8.

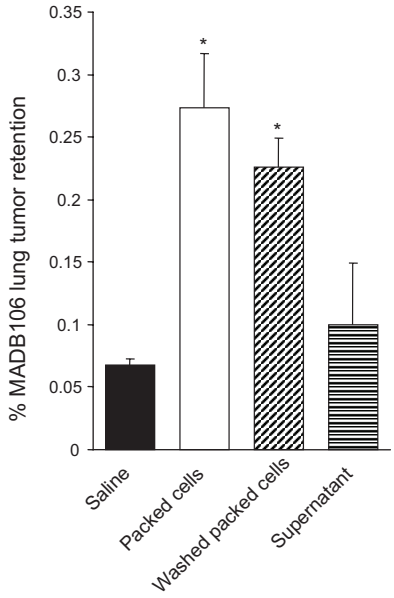

Fig. 3. Cells but not supernatant of stored blood increase MADB106 lung tumor retention. Percentage of lung tumor retention (mean ± SEM) in rats transfused with 14-days-stored packed cells, poststorage washed packed cells, poststorage supernatant from packed cells (supernatant), or saline. n = 22, 4–8 per group. * Significant pairwise difference from the saline control group (protected least significant difference, $P < 0.05$).

Effects of Cellular and Soluble Fractions

To determine whether the effects in the previous two studies were mediated by stored cells of the transfusion or by soluble factors secreted by them, we tested each component separately. Aliquots of allogenic packed cells stored for 14 days were used to extract the supernatant or the cellular fraction without the supernatant (after three washes) or were reconstituted in saline before transfusion (packed cells). The results indicated that packed cells as well as the washed cellular fraction (washed packed cells) significantly increased percentage lung tumor retention ($P < 0.05$) more than threefold (*e.g.*, from saline: 0.068 ± 0.005 to washed packed cells: 0.226 ± 0.049; $P < 0.05$), whereas soluble factors in the supernatant had no deleterious outcome (fig. 3). The study was replicated twice, yielding the same results.

Erythrocytes but Not Leukocytes Mediate the Impact of Blood Transfusion on MADB106 Lung Tumor Retention

To assess the role of erythrocytes and distinguish it from that of leukocytes, we used three approaches. In the first, we conducted leukoreduction in some but not in other allogeneic blood samples before a 14-day storage period. Increasing quantities of leukoreduced erythrocytes and similarly handled nonleukoreduced packed cells were transfused and compared with saline and with untouched packed cells. The results clearly indicated that the number of leukocytes was not the factor that predicted the deleterious effects of the transfusion, but

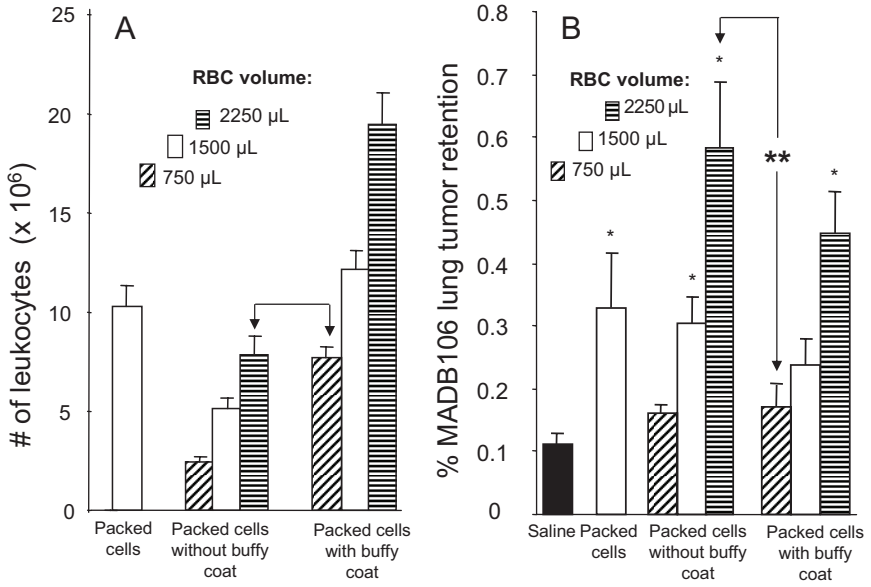

Fig. 4. Erythrocyte (RBC) cell volume, but not leukocyte number, determines the metastasis promoting effects of the transfusion. (*A*) Numbers (mean ± SEM) of leukocytes per a transfusion containing 0, 0.75, 1.5, or 2.25 ml RBCs (diluted in saline to a standard 3-ml volume). Transfusions were conducted after 14-day storage of leukoreduced or nonleukoreduced packed cells. Notice that the two groups contrasted by the *horizontal bar* have similar numbers of leukocytes but different RBC volumes. (*B*) Percentage of MADB106 lung tumor retention (mean ± SEM) in the different groups. Notice that the same two groups contrasted are significantly different from each other in tumor retention (, protected least significant difference, $P < 0.05$), although they have the same number of leukocytes (*A*). * Significant pairwise difference from the respective saline control group (protected least significant difference, $P < 0.05$). n = 67; 6–10 per group.**

rather the volume of transfused erythrocytes, irrespective of whether leukoreduction was conducted. Most illustrative is the comparison between the transfusion of the highest erythrocyte volume in the leukoreduction groups and the transfusion of the lowest erythrocyte volume in the nonleukoreduction groups (indicated in fig. 4 by the horizontal lines). These two groups had the same number of leukocytes (fig. 4A), but the first showed a fivefold increase in lung tumor retention (from saline: 0.113 ± 0.025 to 2,250 µl: 0.584 ± 0.103; $P < 0.05$), whereas the second showed a nonsignificant 1.3-fold effect (to 0.153 ± 0.048; fig. 4B). These findings suggest that erythrocyte volume, rather than leukocyte number, determine the deleterious effects we observed thus far.

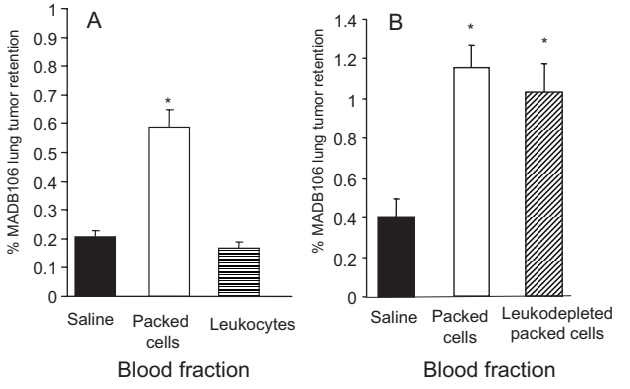

Fig. 5. Leukocytes do not increase MADB106 lung tumor retention, whereas leukodepleted erythrocytes (leukodepleted packed cells) do increase it. Percentage of lung tumor retention (mean ± SEM) in rats transfused with: (*A*) Packed cells, but not leukocytes, significantly increased tumor retention. n = 24; 6–9 per group. (*B*) Both packed cells and leukodepleted packed cells (only erythrocytes) significantly increased tumor retention. n = 21; 6–8 per group. * Significant pairwise difference from the respective saline control group (protected least significant difference, $P < 0.05$).

To further negate the role of leukocytes, we used a second approach in which we directly assessed the impact of isolated allogeneic leukocyte transfusion. Leukocytes were separated before a 14-day storage period and were compared with similarly stored packed cells and with saline. Fluorescence-activated cell-sorter analysis indicated that the transfused leukocytes contained all their subpopulations. The results indicated that stored leukocyte transfusion did not cause any increase in percentage lung tumor retention (fig. 5A), whereas packed cells caused an approximately threefold increase (from saline: 0.205 ± 0.023 to packed cells: 0.588 ± 0.060; $P < 0.05$). These findings negate a role for stored leukocytes (or factors they secrete) in mediating the metastasis-promoting effects evident herein.

Last, to validate the critical role we ascribed to erythrocytes, we took a third approach that relies on administering erythrocytes thoroughly depleted of leukocytes. To this end, we used the standard clinical procedure of filter-based prestorage leukodepletion that eliminated more than 99.99% of leukocytes (see Materials and Methods) and compared it with packed cells and with saline transfusion. The results indicated that both the leukodepleted erythrocytes (mean = 1.036 ± 0.152) and the packed cells (mean = 1.173 ± 0.124) caused approximately threefold increase in percentage lung tumor retention compared with saline (mean = 0.398 ± 0.121; $P < 0.05$; fig. 5B).

Effects of Erythrocyte Transfusion in the Context of Surgery and Blood Loss

To start testing the relevance of our finding to the clinical setting, we also studied the impact of stored erythrocyte transfusion in the context of surgery and blood loss. Animals either underwent laparotomy or were only anesthetized, and were further subdivided and

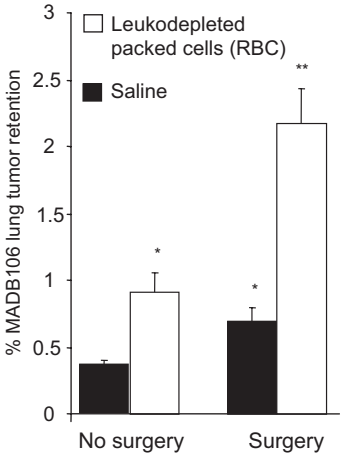

Fig. 6. Stored leukodepleted packed cells (erythrocytes [RBCs]) increase MADB106 lung tumor retention in operated rats more than in nonoperated rats. Percentage of lung tumor retention (mean ± SEM) in rats undergoing or not undergoing surgery and transfused with stored leukodepleted packed cells (RBCs) or with saline. Surgery significantly increased percentage of lung tumor retention, and blood transfusion further increased it, causing a significantly greater impact in operated than in nonoperated animals. * Significant pairwise difference from the no surgery saline group (protected least significant difference, $P < 0.05$). ** Larger effect of RBC transfusion in animals undergoing surgery, compared with no surgery animals (analysis of variance interaction, $P < 0.05$). n = 44; 7–9 in no surgery groups, 14–16 in surgery groups.

transfused during surgery/anesthesia with prestorage (14-day) leukodepleted packed cells (only erythrocytes) or with saline. Just before transfusion, 1 ml blood was withdrawn from all rats by cardiac puncture. The results indicated that erythrocyte transfusion increased MADB106 lung tumor retention in both operated and nonoperated rats (fig. 6). In fact, beyond the already elevated risk caused by surgery itself (from 0.356 ± 0.041 to 0.702 ± 0.125), erythrocyte transfusion caused greater increase in lung tumor retention in operated rats (mean = 2.169 ± 0.316) compared with nonoperated rats (mean = 0.912 ± 0.158), as indicated by a significant interaction between blood transfusion and surgery (two-way analysis of variance indicated significant main effects of surgery [$F_{1,40} = 13.3$, $P < 0.05$] and of transfusion [$F_{1,40} = 21.2$, $P < 0.05$], and a significant interaction [$F_{1,40} = 4.3$, $P < 0.05$]).

Effect on Survival in the CRNK-16 Tumor Model

To investigate the effects of blood transfusion in the CRNK-16 tumor model, allogeneic blood was either separated to the erythrocyte and the leukocyte fractions or was not separated, and was stored for 14 days as packed cells. Rats were transfused with stored leukocytes, stored packed erythrocytes, or stored packed cells, and were compared with rats transfused with fresh syngeneic (autogenic) packed cells and with rats not transfused. Each transfusion originated from 6 ml blood and was given in the standard 3-ml volume. The results indicated that both erythrocyte and packed cell transfusion significantly reduced survival rates ($P < 0.05$; fig. 7). On the other hand, the leukocyte transfusion group was similar to the two control groups (fresh autologous transfusion and no transfusion), falling between them. These findings using the CRNK-16 leukemia model support our previous results in the MADB106 model indicating that the stored erythrocytes, but not stored leukocytes (or substances they secrete), are responsible for the deleterious effects of stored blood transfusion.

Discussion

The leading hypothesis regarding the deleterious impact of blood transfusion on cancer progression considers allogeneic leukocytes to be the major mediating agent.[7,11] Accordingly, leukodepletion is a common prophylactic procedure.[7] Here, for the first time, we show

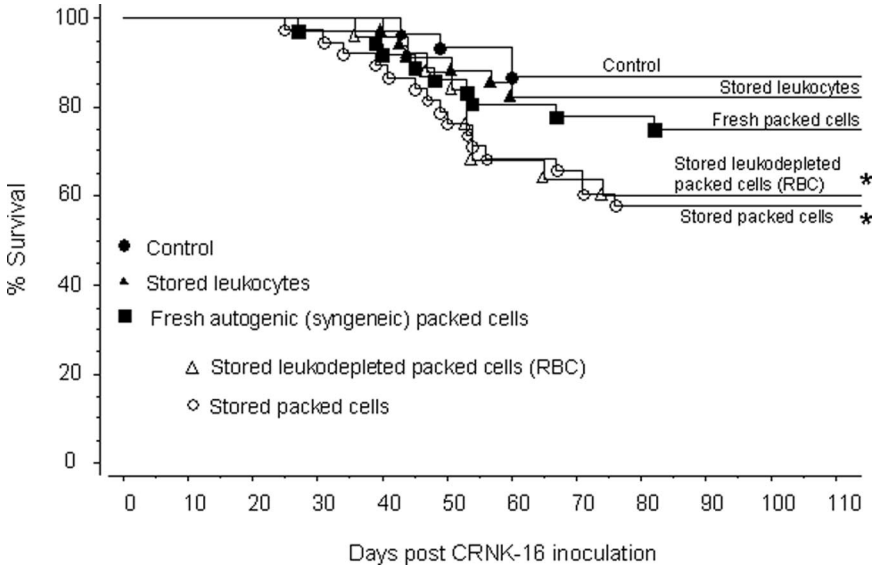

Fig. 7. Transfusion of stored blood, specifically stored erythrocytes (RBCs), reduced survival rates in CRNK-16–derived leukemia. Transfusion of stored packed cells and transfusion of stored leukodepleted packed cells (only RBCs) significantly reduced survival rates (Tarone–Ware test, $P < 0.05$), whereas transfusion of leukocytes or fresh autogenic packed cells had no effect. * Significant pairwise difference from the control group. n = 163; 28–39 per group.

that in two animal models, donors' erythrocytes, rather than leukocytes or soluble factors, can be a critical constituent underlying the cancer-promoting effects of blood transfusion. We further show that these effects of erythrocytes critically depend on the duration of blood storage, irrespective of donors' histocompatibility. Specifically, in our models, both autologous and allogeneic blood transfusions increased cancer progression when stored for more than 9 days, whereas fresh blood, allogeneic or syngeneic, had no deleterious effects. Storage is known to result in the deterioration of erythrocytes,[21] which we believe substantially contributed to their evident cancer-promoting effects.

The medical context of blood transfusion in cancer patients and the involvement of immunity in the potential deleterious effects of the transfusion should be considered when evaluating the clinical relevance of this study. In cancer patients, blood is often transfused around the time of excising the primary tumor. As was recently reviewed by others and us, this perioperative period is characterized by numerous processes that induce an abrupt elevation in the risk for the outbreak of preexisting micrometastases and the seeding of new metastasis.[22] Most relevant to our framework, malignant tissue is notoriously noncohesive, and surgical procedure often disrupts the neoplasm or its vascularization, leading to a release of tumor cells into the circulation.[23,24] As a matter of fact, recent studies indicate that most cancer patients harbor single cancer cells after the removal of the primary tumor.[25] In the current study, the two tumor models used simulate the presence of circulating cancer cells, as well as a sudden elevation in the risk of cancer progression in conjunction with blood transfusion. A second aspect of tumor–host interaction that is relevant to the context of blood transfusion is the role of cellular immunity, particularly T cells and NK cells, in controlling minimal residual disease.[25] Many primary tumors evolve to become nonimmunogenic, or develop other escape mechanisms to evade adaptive immunity. Cytotoxic T cells and NK cells have been reported to interact with the malignant tissue along this process, to restrict metastases, and to play a role in eradicating residual cancer after the primary tumor has been removed.[26–29] In the current study, the two tumor models used are nonimmunogenic and are sensitive to the *in vivo* activity of NK cells,[15,17–19] an important aspect of innate immunity. Further supporting the potential role of NK cells in the current study are the findings that the cancer-promoting effects commence immediately upon erythrocyte transfusion and without previous exposure to the tumor. Therefore, if immunity is involved, it is innate immunocytes, which can react immediately upon first encounter with tumor cells.[16] Overall, the tumor models used herein and the study of erythrocyte transfusion to operated rats reflect important aspects characterizing the clinical setting of blood transfusion in cancer patients, and the potential relevance of innate cellular immunity (*e.g.*, NK cells) in eliminating residual cancer cells.

It is questionable whether tumor models, even the most advanced, can indeed simulate the prolonged and complex processes of human cancer evolvement and interactions with the patient immune system and other physiologic constituents. Therefore, animal studies using tumor models can only suggest mechanisms and potential clinical outcomes, and these suggestions should eventually be tested in humans. Each of the tumor models that we have used has its advantages and shortcomings. The MADB106 experimental metastasis model does not entail a primary tumor, hence its shortcoming as a model. However, MADB106 tumor cells are well characterized with respect to susceptibility to specific immunocytes (*e.g.*, NK cells)[15,17,18] and the time course of this susceptibility (up to 24 h after inoculation).[17] Therefore, it is used herein to suggest immune mediation of the effects and their exact time dependency. This model is also used to simulate the final stages of tumor extravasation and survival in a target organ. In fact, the lung tumor retention of MADB106 cells is an early indicator of the number metastases to be formed in the lungs weeks later, as indicated with respect to the effects of NK depletion, ethanol intoxication, adrenergic and prostaglandin challenges, and the impact of surgery.[15,17,18,30–32] The second tumor model used herein, the CRNK-16 leukemia, may be considered as having greater clinical relevance with respect to blood-borne tumor progression. The CRNK-16 line originated from a naturally occurring leukemia that is highly malignant and is the major cause of natural death in senescent F344 rats. As is common in many human malignancies, this tumor expresses low levels of major histocompatibility complex I, and does not evoke effective immunologic memory.[19] Therefore, the herein orthotopic implantation of CRNK-16 tumor cells enable the *in vivo* study of cancer progression in a biologically relevant setting, which may also bear clinical significance.

Stored or deteriorating erythrocyte transfusion may impact blood-borne cancer progression *via* numerous mechanisms, immunologic or nonimmunologic. The involvement of host immune mechanisms was suggested by several studies,[9,33] but the specific mediating potential of donor erythrocytes was not addressed. It could be hypothesized that deteriorating erythrocytes, which were reported to alter membrane determinants,[21] may preoccupy host innate immune effector cells, leaving tumor cells unattended. Provided that as few as 0.1% of the transfused erythrocytes would become targets to host immunocytes, these erythrocytes will outnumber residual/circulating tumor cells by several orders of magnitude and will probably outnumber relevant host immunocytes. This would dramatically reduce the chances that a host immunocyte would interact with a residual

tumor cell and eliminate it. A nonexclusive hypothesis may address the role of host cytokines and hormones. Deteriorating erythrocytes are approached and eliminated by host splenic and hepatic leukocytes that also control the host milieu of various soluble factors.[34] Specifically, after blood transfusion there is a reduction in splenocyte secretion of interleukin 2 and elevation in monocyte-derived systemic levels of prostaglandin E2.[35,36] Both perturbations are known to suppress cellular immunity, NK cells in particular.[30,37] Last, it is noteworthy that both hypotheses presented in this paragraph are equally relevant to autologous and allogenic deteriorating erythrocytes, which indeed did not differ in their deleterious effects in the current study.

Several limitations of our study should be considered. As already noted, animal models of cancer cannot be expected to simulate the much longer and perhaps more complicated course of cancer evolvement and progression in humans. The tumor models used herein simulate only some aspects of human host–cancer interactions, involving some, but not other, relevant immune and nonimmune mechanisms. In addition, our outcomes are focused on relatively short-term consequences of blood transfusion, with the exception of survival rates in animals challenged with the CRNK-16 leukemia. With respect to the transfused blood, although both human and rat erythrocytes were reported to deteriorate during storage in citrate–phosphate dextrose solution with adenine, rat erythrocytes exerted quicker alterations in several parameters.[38] Therefore, the exact time course of storage-dependent deleterious effects should be studied in human blood. Last, it was beyond the scope of the current study to reveal molecular mediating mechanisms of the deteriorating erythrocytes in rats or in humans. Therefore, more studies are needed to better understand the phenomenon and devise optimal prophylactic approaches.

The clinical implications of our results could, nevertheless, be significant. Although erythrocyte transfusion cannot be altogether avoided, the use of freshly drawn blood should be tested in cancer patients. Further research in animal models and in cancer patients is required to determine mediating mechanisms, specifically the role of host innate immunity, the cytokine response, and nonimmunologic mechanisms. Other animal studies implicated allogenic leukocytes in promoting cancer progression. However, unlike in the current study, in these earlier studies, blood was transfused several days before or several days after exposure to the tumor,[9,10] and therefore, these earlier studies are likely to reflect different mediators. Taken together, different mechanisms could be involved and may impact different aspects of host–tumor interactions in different time frames. Irrespective of mediating mechanism, we suggest that donors' leukocytes might not be responsible for every adverse outcome of blood transfusion, and that the universal procedure of leukodepletion may not be sufficient to overcome the deleterious effects of blood transfusion in cancer patients. Long-stored erythrocytes should be studied as a potential risk factor in cancer and noncancer patients. A combined approach using leukodepletion in freshly drawn blood may prove to be a safer alternative in the context of oncologic surgeries, as well as in other surgeries involving high risk of infection and complications, and therefore deserves further clinical studies.

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