Published in final edited form as:
J Trauma. 2009 October ; 67(4): 792–801. doi:10.1097/TA.0b013e31819d9c04.

Hemorrhage and Subsequent Allogenic Red Blood Cell Transfusion are Associated With Characteristic Monocyte Messenger RNA Expression Patterns in Patients After Multiple Injury—A Genome Wide View

Viktoria Bogner, MD, **Henry V. Baker, PhD**, **Karl-Georg Kanz, MD**, **L. L. Moldawer, PhD**, **Wolf Mutschler, MD**, and **Peter Biberthaler, MD**

Chirurgische Klinik und Poliklinik-Innenstadt, Ludwig-Maximilians University, Munich, Germany (V.B., K.-G.K., W.M., P.B.); Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Gainesville, Florida (H.V.B.); and Department of Surgery, Laboratory of Inflammation Biology and Surgical Science, University of Florida College of Medicine, Shands Hospital, Gainesville, Florida (L.L.M.).

Abstract

Introduction—As outcome to severe trauma is frequently affected by massive blood loss and consecutive hemorrhagic shock, replacement of red blood cell (RBC) units remains indispensable. Administration of RBC units is an independent risk factor for adverse outcome in patients with trauma. The impact of massive blood transfusion or uncrossmatched blood transfusion on the patients' immune response in the early posttraumatic period remains unclear.

Material—Thirteen patients presenting with blunt multiple injuries (Injury Severity Score >16) were studied. Monocytes were obtained on admission and at 6, 12, 24, 48, and 72 hours after trauma. Biotinylated complementary RNA targets were hybridized to Affymetrix HG U 133A microarrays. The data were analyzed by a supervised analysis based on whether the patients received massive blood transfusions, and then subsequently, by hierarchical clustering, and by Ingenuity pathway analysis.

Results—Supervised analysis identified 224 probe sets to be differentially expressed ($p < 0.001$) in patients who received massive blood transfusion, when compared with those who did not. In addition, 331 probe sets were found differentially expressed ($p < 0.001$) in patients who received uncrossmatched RBC units in comparison with those who exclusively gained crossmatched ones. Functional pathway analysis of the respectively identified gene expression profiles suggests a contributory role by the AKT/PI3Kinase pathway, the mitogen-activated protein-kinase pathway, the Ubiquitin pathway, and the diverse inflammatory networks.

Conclusion—We exhibited for the first time a serial, sequential screening analysis of monocyte messenger RNA expression patterns in patients with multiple trauma indicating a strongly

Address for reprints: Viktoria Bogner, MD, Chirurgische Klinik und Poliklinik-Innenstadt, Ludwig-Maximilians University, Munich, Nussbaumstr. 20, Munich 80336, Germany; Viktoria.Bogner@med.uni-muenchen.de..

significant association between the patients' genomic response in blood monocytes and massive or uncross-matched RBC substitution.

Keywords

Microarray; MAP kinase; Ubiquitin; Allogenic red blood cell transfusion; Hemorrhagic shock

The initial posttraumatic resuscitation management of patients with multiple trauma presents a number of ambitious challenges. As the clinical outcome of patients with multiple injuries is frequently threatened by massive blood loss and consecutive hemorrhagic shock, a major task lies in the rapid substitution of not only the lost intravascular volume but also the oxygen carriers.[1] Following actual clinical guidelines, the common clinical practice for therapy of hemorrhagic shock involves fluid resuscitation and the application of allogenic red blood cell (RBC) units. By default under emergency conditions, clinical practice in Europe and the United States permits the application of uncrossmatched type-O RBC units (UORBC).[2,3] To give a dimension to the size of the problem, an estimated 10% to 15% of the total RBCs administered in the United States are used for the treatment of patients with injuries.[4] According to the German Trauma Society, 45% of all patients with severe trauma exhibiting an Injury Severity Score (ISS) >16 points, receive blood transfusion during their resuscitation period.[5] Although being conscious of the lifesaving nature and indication of RBC application in emergency situations, the risks and side effects of massive and uncrossmatched RBC resuscitation are substantial (for review see Ref. 6). Besides the low but inherent risk of viral transmission with HIV, Hepatitis B virus, or Hepatitis C virus, there is a strong association between blood transfusion and early immunomodulation[7–9] promoting the development of a systemic inflammatory response syndrome (SIRS)[10–12] and subsequently multiple organ failure (MOF).[13,14] Most importantly, blood transfusion is identified as an independent risk factor for adverse posttraumatic outcome.[11,12] To date, there is very little information about the pathophysiological mechanisms and functional pathways being associated to massive RBC transfusion that lead to the development of the aforementioned complications such as SIRS, MOF, and death at the worst. Especially in this context, peripheral blood monocytes as elements of the innate immune system seem to play a pivotal role.[15,16] To further elucidate the impact that hemorrhage and consecutive allogenic blood transfusion have on an immune-competent cell population, we initiated in accordance with our previous work[17,18] a genome-wide exploration of the monocyte transcriptome in patients with multiple injuries who received massive blood transfusions (>10 RBC units in the first 24-hour posttrauma), when compared with those who had received less than 10 RBC units. To clarify the influence of crossmatching, the gene expression patterns from patients who had received UORBC were compared with those from patients after application of exclusively crossmatched RBC units.

PATIENTS AND METHODS

Study Design and Patient Collective

After permission by the Ethical Committee at Ludwig Maximilians University (reference number: 012/00), the study was performed following Good Clinical Practice at our Level I trauma center. Approval was also granted by the Institutional Review Board of the

University of Florida College of Medicine concerning the processing and analyses of these anonymous blood samples generated from the completion of this study in Munich. Patients of age with multiple injuries (ISS of >16 points[19]) who reached the emergency department within 90 minutes after the traumatic event were included. Signed informed consent was obtained from the patient or from a legal representative. Patients who did not survive the first 24-hour posttrauma were excluded. After the initial resuscitation and primary surgical interventions in accordance with standard of care, patients were admitted to the surgical intensive care unit. RBC unit resuscitation already started in the emergency room. Patients were assigned into different clinical groups according to the following two criteria of transfusion: (i) amount of RBC substitution differentiating patients who underwent massive transfusion defined by the application of more than 10 RBC units during the first 24 posttraumatic hours[4] and such who gained less (group 1: patients who received >10 RBC units vs. group 2: patients who received <10 RBC units/24 h) and (ii) manner of blood transfusion respecting whether applied RBC units were crossmatched with the patients' blood group or whether the patient had received UORBC units (blood group 0, cc dd ee) at any time in the first 72 hours after trauma (group 3: patients after UORBC units vs. group 4: patients after crossmatched RBC units only). Additionally, fresh frozen plasma (FFP) unit application was also recorded. However, the amount of FFP application did not influence the patients' grouping into the different clinical blood transfusion classes. To assess the depth of shock and resulting acid-base imbalances, base excess (BE) levels, systolic blood pressure, and shock-index (heart rate/systolic blood pressure) have been recorded for the respective clinical groups at every blood sampling time point.

Blood Sampling

A total volume of 30 mL of ethylenediamine tetraacetic acid-anticoagulated whole arterial blood each was drawn on admission within 90 minutes after the traumatic event and at 6, 12, 24, 48, and 72 hours after the trauma. After RBC lysis, the resulting leukocyte pellet was resuspended in phosphate saline/ethylenediamine tetra-acetic acid buffer for monocyte separation using anti-CD 14 antibody coated micromagnetic beads (Miltenyi Biotech, Auburn, CA) in accordance to the manufacturer's instructions. The purity of the isolated monocyte fraction was documented using fluorescence activated cell sorting analysis in blood samples from healthy subjects. Total monocyte RNA preparation was performed by RNeasy Midi Kit following the supplier's (Qiagen) recommendations, completed by an additional on column DNase digestion step (MiniElute Clean Up Kit, Qiagen). RNA quality and purity were assessed by capillary electrophoresis (Bioanalyzer 2001, Agilent, Palo Alto, CA) before and after the first and second round of amplification.

Complementary RNA Synthesis and Microarray Hybridization

Complementary RNA synthesis was performed from 0.4 μg of total RNA using a two-step amplification protocol based on the supplier's recommendations (Affymetrix, High Wycombe, UK) with the exception that an ENZO Bio Array High Yield RNA Transcript Labeling Kit (T7, Enzo Life Science, Farmingdale, NY) was used for in vitro complementary RNA transcription and incorporation of biotinylated nucleotides. The resulting product was then hybridized onto an Affymetrix HGU133A GeneChip (Affymetrix, 14,500 genes and 22,283 probe sets) for 16 hours at 45°C in an Affymetrix

hybridization oven. The Affymetrix fluidics station was used for staining and washing according to the Affymetrix protocol (EukGEWSv4). The Arrays were scanned with an Affymetrix scanner.

Microarray Data Analysis and Biostatistics

Low-level analysis was performed using the perfect match algorithms in DChip (Wong Laboratory, Department of Biostatistics, Harvard School of Public Health, USA) (for review see Ref. 20). Background intensity was calculated by Affymetrix Microarray Suite version 5.0 (MAS5.0). Probe sets exhibiting hybridization intensity levels below or at background intensity on all arrays were considered as to be “absent” and were excluded from further data analysis. High-level statistical analyses such as clustering or prediction modeling were calculated using algorithms contained in DChip and BRB Array Tools (for details see http://linus.nci.nih.gov/BRB-ArrayTools.html[21]).

Supervised Analysis and Prediction Models

The aim of the supervised analysis was to identify genes, which might serve as class identifiers for massive blood transfusion (more than 10 RBC units in the observation period) or for the application of UORBC units at any time in the observation period. In both cases, arrays were assigned to one of two clinical entities according to the aforementioned criteria, whereas patients in clinical group 1 had received massive transfusion in contrast to those in clinical group 2. Patients assigned to group 3 had received at least one UORBC unit during the first 72-hour posttraumatic hours as opposed to patients in clinical group 4 who were only applied crossmatched RBC units. Probe sets whose hybridization signal intensities differed between the respective opposed two groups at the $p < 0.001$ level of significance were identified. The ability of these probe sets to function as a classifier for transfusion manner or extent was established using the support vector machine prediction model and leave one out crossvalidation (LOOCV).[21] To determine whether the misclassification rate observed was below the rate expected by chance alone, Monte Carlo simulations were performed on 2,000 random permutations of the dataset.

Pathway Analysis

After the identification of probe sets, which exhibited significantly different gene expression profiles depending on the patients’ blood transfusion status, we first intended to identify functional groups of genes being overrepresented in the present dataset. Second, we examined whether the expression responses may have affected any specific signaling pathways or biological networks. Therefore, the Ingenuity pathway analysis program (Ingenuity Systems, Mountain View, USA, www.ingenuity.com) was run with the probe sets significant at $p < 0.001$ concerning the respective transfusion criterions. Based on an extensive scientific knowledge database respecting biological interactions among, for example, transcription factors, enzymes, receptors, mediators, etc., this software suggests and visualizes functionally different relationship networks among the gene products of the disposed probe sets. One network contains up to 35 factors that are encoded by the respecting probe sets. To assess whether there are different gene subnetworks found to be overrepresented depending on the patients’ clinical group, the characteristic gene expression

profiles were compared with each other. Duplicate genes, which are differentially expressed in both blood transfusion analyses, were identified.

RESULTS

Patient Collective and Clinical Data

Thirteen patients (11 men and 2 women) fulfilled the entry criteria and were enrolled in the study. Thereof, three deceased within 90 days after the traumatic event. Patients' ages ranged from 19 to 65 years, and the ISS ranged from 19 to 75 points with a mean (±SD) of 40 (±14). Four patients (clinical group 1: "massive transfusion") required massive RBC substitution during the observation period, the mean (±SD) of applied RBC units in this group counted 25.5 (±6.1) units (300 mL leukocyte depleted, packed RBCs, each) and 25.2 (±8.5) units of FFP, whereas nine patients (clinical group 2: "moderate transfusion") received 4.1 (±3.6) RBC units and 4.8 (±5.8) FFP units, mean (±SD). For the sake of emergency, 7 of the 13 investigated subjects needed to be initially provided with UORBC (clinical group 3: "UORBC"), whereas six patients only received crossmatched and thereby compatible blood (clinical group 4: "crossmatched transfusion").

Patients who require massive RBC transfusion (group 1) exhibit a significant higher BE on admission (0 hour: $p < 0.014$ *t* test), at 12 hour; however, the tendency switches and BE in these patients is lower than in such without massive transfusions (24 hours: $p < 0.001$ *t* test; 48 hours: $p < 0.01$, *t* test). Clinical group 1 also shows a significantly lower systolic blood pressure on admission (*t* test, 0 hour: $p < 0.031$) and correspondingly higher shock index at 0, 12, and 24 hours (*t* test, 0 hour: $p < 0.001$, 12 hours: $p < 0.028$, 24 hours: $p < 0.026$). Patients who had received uncrossmatched RBC units only significantly differ on the admission time point concerning two clinical criteria (*t* test, BE 0 hour: $p < 0.022$, systolic BP 0 hour: $p < 0.029$).

More detailed clinical baseline characteristics are summarized in Table 1.

Supervised Analysis

We first intended to determine whether massive blood transfusion generally provokes specific gene expression patterns in circulating human monocytes. The arrays were appointed to one of the respective clinical groups depending on the amount of RBC-unit transfusion, subsequently a modified students *t* test was performed to identify probe sets whose hybridization differed between the two groups at $p < 0.001$. Two hundred twenty-four probe sets (representing 206 genes) exceeded that threshold. Hierarchical clustering of these genes visualizes the gene expression differences according to the different clinical groups (Fig. 1). There is a cluster containing almost exclusively arrays gained from patients of clinical group 1, whereas arrays from patients with moderate RBC requirement (group 2) separate in another dendrogram on the left. To quantify the potential of the 224 probe sets as classifiers for blood transfusion dimension, we calculated a biostatistical prediction model based on a LOOCV algorithm (BRB Array Tools, for review see Ref. 21). This statistical approach had already proven of value in our precedent study where we identified gene

expression classifiers for outcome and clinical entities.[18] Prediction model calculation revealed 95.4% classification sensitivity and 95.6% classification specificity.

In a second step, we analyzed whether the criterion "uncrossmatched blood transfusion" equally affects particular gene expression patterns. Figure 2 shows the expression of 331 probe sets (representing 300 genes) being significantly different expressed ($p < 0.001$ significance level in a students *t* test between the two clinical groups) depending on whether the patients underwent uncrossmatched blood transfusion (group 3) or were exclusively resuscitated using crossmatched RBC units (group 4). Similar to the "massive transfusion criterion," hierarchical clustering again reveals two separate cluster dendrograms, the right one is mostly composed of arrays from patients with exclusively crossmatched RBC-unit application, in contrast, arrays clustering in the left dendrogram mainly belong to patients who gained also UORBC units (Fig. 2). Regarding the criterion "application of UORBC-units," computation of the support vector machine prediction model showed 97.2% sensitivity and 96.8% specificity.

Additionally, Monte Carlo simulations calculating 2,000 random permutations were performed to assure that the misclassification rates we observed in the crossvalidation study were significantly less than could be expected by chance alone.[22] This was indeed the case in all our LOOCV calculation.

Finally, we compared the identified probe sets differentiating massive transfusion and UORBC transfusion with each other. The UORBC analysis has 81 probe sets in common with the massive transfusion experiment, this complies 25% of the total probe sets significant for this setting (the duplicates are listed in supplemental Table 1, Duplicate Genes in Massive Transfusion and UORBC Transfusion, on our homepage http://chirinn.klinikum.uni-muenchen.de/forschung/fors_01_14_ 03_03.html).

Pathway Analysis

Massive Blood Transfusion—The Ingenuity pathway analysis tool focused 156 of the total 224 probe sets that are differentially expressed in patients with massive transfusion compared with patients with less RBC requirement.

Top Functional Groups: The products of these encoding pathway genes are involved in essentially three top functional components: First, "immune response" including elements of the interleukin-6 and interleukin-10 signaling pathway. Second, "cellular movement" and third, "hematological system development and function," especially managing the recruitment, chemotaxis, migration, and accumulation of immune-competent cells such as neutrophils, lymphocytes, monocytes, or myeloid dendritic cells, such as the chemokines CXCL2, CXCL3, or the surface receptor CD86 and the platelet activating factor, for example, acetylhydrolase PAFAH1B1.

Ubiquitin Pathway: Beyond this, hemorrhage and subsequent massive blood transfusion are associated with an overexpression of the Ubiquitin-Pathway including Ubiquitin C and a row of factors involved in the connected regulatory network such as transcription elongation factor, the two peptidases proteasome activator subunit 2, proteasome subunit beta type 9

involved in immune system-related proteolysis and the ubiquitin-conjugating enzyme UBE2D2, a protein ligase. All these factors are part of the protein ubiquitination process, a major regulatory mechanism of protein degradation and quality control.

Mitogen-Activated Protein-Kinase Pathway: Figure 3 demonstrates one network of 17 genes and their functional relationships around the transcription factor JUN, activating others such as the transcriptional repressor cAMP responsive element modulator, the interleukin 1β-regulated MAF-F (v-MAF musculoaponeurotic fibrosarcoma oncogene) and the enzyme Harvey rat sarcome viral oncogene homolog, an immune modulator on multiple levels. Ingenuity interestingly identified a connection between the latter with the thrombin-activating gene mitogen-activated protein-activated protein kinase 2 (MAPKAPK2) and again with proteasome subunit beta type 9 and proteasome activator subunit 2. In addition, JUN-overexpression associates activation of lysosomal-associated menbrane protein 2, which is known to initiate pathologic platelet activation. JUN itself, in combination with MAPKAPK2, v-ets erythroblastosis virus E26 oncogene homolog and Harvey rat sarcome viral oncogene homolog furthermore builds a connection to the MAP-kinase pathway family.

Uncrossmatched Transfusion—Concerning those probe sets, which serve as classifiers for the donation of UORBC units, Ingenuity considered 212 of total 331 probe sets as focus genes for the analysis.

Top Functional Groups—As in the latter analysis, the three top-represented gene function groups again include “immune response,” “cellular movement,” and “development and function of the hematological systems,” whereas these tasks are partly covered by the same genes, partly by different ones. For example, PECAM-1 (platelet/endothelin cell adhesion molecule), PF4 (platelet factor 4), xand v-ets erythroblastosis virus E26 oncogene homolog are also found to be differentially expressed in both settings. A detailed list of the duplicate genes and their Affymetrix identification number will be published on our homepage http://chirinn.klinikum.uni-muenchen.de/forschung/fors_01_14_03_03.html.

Inflammation—The pathway analysis system furthermore reveals another interesting network (Fig. 4) that mainly consist of genes also responsible for immunologic activation, for example, the proinflammatory interleukin-8 and the antiinflammatory interleukin-10. They both are closely interacting with interleukin-1-receptors (Il-1RN and Il-1R1) and with the monocyte CD 14 receptor.

PIK3/AKT Kinase Pathway—In contrast to the massive transfusion gene set, patients who received uncrossmatched transfusion exhibit an upregulation of the factors growth factor receptor bound protein, phosphatidylinositol-3 kinase, regulatory subunit 1 (PIK3R1), and gene for the inhibitor of NFKB and IkB (NFKBIA). Those three build a connection between a G-Protein-Coupled Receptor Signaling Pathway involving the Dual Specificity Phosphatase 1 (DUSP1), which is acting as a MAPK-Kinase and also connected to the aforementioned MAPKinase-Pathway elements, endothelin receptor type B and calcium/calmodulin-dependent kinase II gamma with the PI3K/AKT (serine/threonine kinase)-Kinase Signaling Pathway—a regulation pathway that is involved in cell cycle progression,

furthermore represented by stratifin, forkhead box O1A, and cyclin-dependent kinase inhibitor 1.

DISCUSSION

Oligonucleotide microarray technology has become a very powerful tool in screening nearly the entire human genome on various research questions in an unbiased manner. Its use gains more and more popularity not only in basic research issues but also the technique has become also a favored method to facilitate the understanding of clinical phenomenon. In a first approach, we conducted a pilot study and analysis in human monocytes of patients after blunt multiple injury to identify genes, which can classify clinical outcome with a high specificity and sensitivity.[17] Purposefully, we initially chose the very straightforward criterion “clinical outcome” to provide evidence that it is generally possible to perform prospective classification of clinical entities using genome-wide gene expression profiling with the Affymetrix HG U 133A chip (22,000 probe sets and 14,500 genes). In this work, we intend to go in a different direction to investigate in more detail clinical factors that might regulate the genomic response to trauma. On the basis of the same dataset, we could now identify genetic subgroups and specific functional pathways, which are differentially expressed depending on the extent and type of RBC transfusion in patients with multiple trauma after hemorrhage.

Study Design and Patient Collective

We intended to identify gene groups, whose expression is specifically associated to massive transfusion in the first 24 posttraumatic hours and the donation of uncrossmatched RBC units. Patients were assigned to the “UORBC transfusion group” if they had received any uncrossmatched RBC unit in the 72-hour observation period. Massive blood transfusion was defined by the application of more than 10 RBC units in the first 24 hours after trauma, according to the work of Como et al.[4] It may be understood as a critical point that we thereby have set two different time point cutoffs. However, we purposefully set the 72 hour instead of 24 hour concerning the application of UORBC units, as we considered that any application within this 72-hour time frame would lead to a foreign antigen contact with the patients immune system and might provoke respective reactions. Moreover, patients who died in the first 24 hours were excluded from further considerations. We consciously set this condition, as the primary target of our work was not to investigate the connection between early posttraumatic survival and blood transfusion requirement but the potential impact of RBC application on the patients’ immune system function. We assume that patients who require massive blood transfusion and, nevertheless, have an adverse outcome within 24-hour posttrauma are likely to have suffered from injuries that have not been compatible with secondary survival anyway. Therefore, these patients were not included in the study.

Regarding the composition of our different clinical groups, we did not especially address to the amount of applied FFP units. However, these allogenic blood products also exhibit an inherent antigenic potential and may trigger inflammatory reactions.[23] In our patient collective, the amounts of given RBC units were closely equal to those of applied FFP units, which means that patients with massive RBC transfusion also required massive FFP

substitution. However, we must admit that patients who were assorted to clinical group 4 (only crossmatched transfusion) did indeed exclusively gain crossmatched RBC units but not only blood group identical FFP units.

Another critical point of this analysis may be the presence of certain covariates such as injury severity and depth of shock. The ISSs are comparable in groups 1 and 2; however, it differs in groups 3 and 4. We assume that this may also be an influencing factor on our identified genes. Nevertheless, only about 10% (31 genes) of the factors that indicate injury severity can be found in the gene expression pattern that distinguishes group 3 from group 4. The gene set that differentiates extent of blood transfusion merely shares five genes with the ISS-indicating set. Although assuming that injury severity or extent of tissue injury might significantly influence gene expression in patients who require RBC transfusion, the actual gene overlapping is rather marginal. Patients with trauma who require blood volume replacement suffer from hemorrhagic shock that leads to compensatory changes in vital parameters such as blood pressure and heart rate and to changes in acid-base balance. Indicators for the latter are the serum BE, systolic blood pressure, or the shock index. Patients who initially require massive RBC resuscitation suffer from a more severe state of shock, expressed by significant differences in BE, systolic BP, and SI in the first 24-hour posttrauma. However, these parameters are—contrary to straightforward criteria such as injury severity, outcome, or total amount of infused RBC units—rapidly changing and quickly responsive to therapeutic measures. Vital parameters respond to catecholamines, blood pH, and BE changes can be buffered. For this reason, we purposefully renounced analysis of gene expression depending on these flexible factors. Nevertheless, this is a limitation of the study, as we are in the main not able to really separate cause and effect. This question can probably not be clarified in an experimental study in humans.

Contamination with foreign immune cells is an important bias as investigating on patients after massive blood transfusion. In our study, patients received leukocyte depleted packed RBC units. These units contain a residual leukocyte population between 0.5 and 0.7×10^6 per unit.[24] Having a medium volume of 300 mL per unit, we can assume a cell concentration of approximately 1.6 cells/μL. Consequently, a person with a normal leukocyte concentration will have 5,000 cells/μL. As the monocyte fraction represents about 10% of the total leukocyte amount, 500 monocytes will be found in 1 μL blood. By extrapolation from a single RBC unit, the theoretically applied number of administered cells would represent 1.6% of the individual monocyte fraction. If we couple this with the assumption, that the response by these donated cells to the injury stimulus is likely to be somewhat similar to the response by the host cells, we therefore considered the risk associated with this bias as to be acceptably low.

Pathway Analysis

We performed pathway analysis to investigate whether the extent or type of blood transfusion in patients with multiple injuries provokes an overexpression of specific gene subgroups or functional classes. Patients who undergo massive blood transfusion are on the one hand naturally exposed to hemorrhagic shock and hypoxia; on the other hand, it is a well-known fact that allogenic blood transfusion leads to modulations of the immune

system. Despite the problem of blood loss per se and the occurrence of hemorrhagic shock, blood transfusion in general is considered as an independent risk factor for the development of a multiple organ failure syndrome and a higher mortality rate.[6,10,11,25]

Top Functional Groups

Comparing the two sets of genes (massive transfusion and UORBC transfusion), the same three top functional gene groups are overexpressed in both analyses. This may partly be rooted in the fact that in both cases, application of UORBC units and massive RBC transfusion always conduces to the therapy of massive blood loss. We thereby consider that some part of the detected differential gene expression will also be an answer to the foregoing stimulus “trauma” or “shock” and not exclusively to the event “blood transfusion.”

A good example for this is the expression of genes managing blood cell recruitment and accumulation such as the chemokines CXCL2 and 3, the platelet/endothelial cell adhesion molecule PECAM-1, or the platelet activating factor acetylhydrolase PAFAH1B1. Interestingly, Aiboshi et al.[13] suspected the platelet activating factor PAF—i.e., connected to the latter mentioned PAFAH1B1—to enhance PMN cytotoxic responses and thereby to increase the risk of postinjury MOF.

Inflammation

As an expression of a general inflammatory reaction after blood transfusion, elements of the proinflammatory Interleukin-6 and Interleukin-8 pathway and of the Interleukin-10 pathway are present in both training sets. This is absolutely in line to other publications and experimental settings, which could prove that the RBC-units application provokes the systemic release of the aforementioned mediators (for review see Refs. 26 and 27) and leads to a higher occurrence of posttraumatic or postoperative MOF[6,27] and a higher susceptibility for infections.[26] Although having some genes and cellular functions in common, only 25% of the differentially expressed genes are total duplicates in the two different analyses. In the following, the differences in gene expression between manner and extent of blood transfusion should be discussed.

Ubiquitin C

Ubiquitin C and the corresponding protein-ubiquitin signaling pathway are upregulated in patients after massive blood transfusion. This is especially evocative, as extracellular ubiquitin and elements of the ubiquitin signaling pathway[28] are not only known to be enhanced in patients after multiple injuries in general,[29] but—if applied for therapeutic reasons—seem to reduce fluid requirements in a experimental hemorrhage mouse model.[30] Furthermore, extracellular ubiquitin seems to have antiinflammatory properties and may be involved in immunosuppressive mechanisms in critical ill patients.[31] Our data support that hemorrhage and massive blood transfusion induces the endogenous ubiquitin production in peripheral blood monocytes, hypothetically in terms of antiinflammation and immunosuppression after increased foreign antigen exposure. This immunosuppression, however, may be one of the causes for a higher infectious susceptibility in patients with trauma after blood transfusion.[26]

PIK3-Kinase/AKT Pathway

In patients who had received uncrossmatched blood transfusion, the NFKBIA (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor) is increased. The gene product of NFKBIA (IKB) is an important inhibitor of NFKB that mediates inflammatory responses on various lanes.[16,31] NFKB inhibition in turn downregulates the expression of multiple proinflammatory genes, diminishes intravascular coagulation, reduces tissue neutrophil influx, and prevents microvascular endothelial leakage (for review see Ref. 32). Thereby, upregulation of NFKBIA seems to be a protective mechanism, which may have the task to counteract posttransfusional overwhelming proinflammatory reactions.

Moreover, NFKBIA gives a link to another pathway upregulated in patients through PIK3R1 and DUSP1: PI3K/AKT-Kinase Signaling Pathway, a regulation pathway that is involved in cell cycle progression, in our data represented by growth factor receptor bound protein 2, PIK3R1, NFKBIA, stratifin, forkhead box O1A, and cyclin-dependent kinase inhibitor 1. AKT pathway enhancement is suspected to mediate cell survival in neuronal cells.[33] Moreover, activation of the PI3K and AKT-Kinase Pathway is associated with increased survival in sepsis.[34] In our case, the PIK3R1, which negatively modulates the catalytic activity of the PIK3[35] is downregulated in patients who had received uncrossmatched blood transfusion. Downregulation of the negative regulator ends up in a stronger activity of PI3-Kinase in this patient subcollective.

MAP-Kinase Pathway

Another attenuation and modulation of the adaptive immune reactions is probably achieved by the differential expression of the Dual Specificity Phosphatases such as DUSP1[36] by regulating various MAP-Kinase proteins. Dong et al.[37] considers in his review that there are three major subfamilies related to the immune system, c-JUN (synonym: ERK), p38MAPK, and JNK (c-Jun N-Terminal Kinases). Interestingly, representatives of all these three subfamilies are found to be differentially expressed in patients after UORBC transfusion. The tasks of these MAP-Kinases in terms of immune response activity are very complex, ending up in up and downregulation of many pro and antiinflammatory cytokines and mediators (for review see Ref. 37).

In our prior analyses, we could identify gene profiles that significantly differentiate various clinical parameters and endpoints. We initially validated the biostatistical system searching for genes that differentiate gender and identified 63 factors that are mainly placed on the y-chromosome. In the next step, we chose the very stringent clinical parameter “outcome” and identified a gene classifier of 763 probe sets that are significantly different expressed in surviving patients, when compared with those who died. We could show that further criteria such as injury severity and the development of MOF also significantly influence gene expression. The literature gives multiple hints that the application of allogenic RBCs is an independent risk factor for the incidence of MOF and adverse outcome in patients with trauma.[11,12] There are some experimental studies that connect single gene products with massive RBC transfusion.[38] However, there is only little information about underlying intracellular mechanisms and pathways that might impact the immune response after hemorrhage and extensive allogenic blood product substitution. We could, using a genome

wide expression study in a limited patient collective, identify gene profiles that are differentially expressed depending on amount and manner of blood transfusion on a high level of significance. Of course, this effect on gene expression level is to be understood as a mixture of different influencing factors such as the trauma itself, depth, and duration of shock. However, these data give hints that hemorrhage and consecutive RBC transfusion is associated with a modification in monocyte gene expression that might lead to a higher degree of inflammation and might trigger SIRS and MOF. This investigation should encourage experimental studies to further verify and clarify the intermediate and long-term effects of allogenic blood products on the developing of patients with multiple trauma.

CONCLUSION

We presented a serial, sequential analysis of initial monocyte messenger RNA expression in patients after multiple injury in the early posttraumatic period using an oligonucleotide microarray platform. We thereby were able to show that characteristic monocyte messenger RNA expression patterns and functional biological networks were noted in patients with multiple injuries with hemorrhage, shock, and blood transfusion. The meaning of these preliminarily identified networks will be the focus of further studies in this field.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge the technical support of the National Institutes of General Medical Sciences and Massachusetts General Hospital who provided technical assistance with the sample preparation, cRNA synthesis, and microarray analysis. Analyses were performed using BRB ArrayTools developed by Dr. Richard Simon and Amy Peng and DChip (Wong Laboratory, Department of Biostatistics, Harvard School of Public Health, USA).

Supported by Large Scale Collaborative Research Program (U54-GM-62119-02), entitled Inflammation and Host Response to Injury (National Institutes of General Medical Sciences; to R.G.T.).

REFERENCES

1. American College of Surgeons. Advanced Trauma Life Support for Physicians: Instructor Manual. American College of Surgeons; IL: 1993. p. 75-110.
2. Dutton RP, Carson JL. Indications for early red blood cell transfusion. J Trauma. 2006; 60:S35–S40. [PubMed: 16763479]
3. Malone DL, Hess JR, Fingerhut A. Massive transfusion practices around the globe and a suggestion for a common massive transfusion protocol. J Trauma. 2006; 60:S91–S96. [PubMed: 16763487]
4. Como JJ, Dutton RP, Scalea TM, Edelman BB, Hess JR. Blood transfusion rates in the care of acute trauma. Transfusion. 2004; 44:809–813. [PubMed: 15157244]
5. Working Group on Polytrauma of the German Society of Trauma Surgery (DGU). Annual Report of the German Trauma Registry. 2003. Available at: www traumaregister de
6. Napolitano L. Cumulative risks of early red blood cell transfusion. J Trauma. 2006; 60:S26–S34. [PubMed: 16763477]
7. Innerhofer P, Tilz G, Fuchs D, et al. Immunologic changes after transfusion of autologous or allogeneic buffy coat-poor versus WBC-reduced blood transfusions in patients undergoing arthroplasty. II. Activation of T cells, macrophages, and cell-mediated lympholysis. Transfusion. 2000; 40:821–827. [PubMed: 10924610]

8. Fransen E, Maessen J, Dentener M, Senden N, Buurman W. Impact of blood transfusions on inflammatory mediator release in patients undergoing cardiac surgery. Chest. 1999; 116:1233–1239. [PubMed: 10559080]
9. Avall A, Hyllner M, Bengtson JP, Carlsson L, Bengtsson A. Postoperative inflammatory response after autologous and allogeneic blood transfusion. Anesthesiology. 1997; 87:511–516. [PubMed: 9316954]
10. Shander A. Emerging risks and outcomes of blood transfusion in surgery. Semin Hematol. 2004; 41:117–124. [PubMed: 14872432]
11. Dunne JR, Malone DL, Tracy JK, Napolitano LM. Allogenic blood transfusion in the first 24 hours after trauma is associated with increased systemic inflammatory response syndrome (SIRS) and death. Surg Infect (Larchmt). 2004; 5:395–404. [PubMed: 15744131]
12. Malone DL, Dunne J, Tracy JK, Putnam AT, Scalea TM, Napolitano LM. Blood transfusion, independent of shock severity, is associated with worse outcome in trauma. J Trauma. 2003; 54:898–905. [PubMed: 12777902]
13. Aiboshi J, Moore EE, Ciesla DJ, Silliman CC. Blood transfusion and the two-insult model of post-injury multiple organ failure. Shock. 2001; 15:302–306. [PubMed: 11303730]
14. Ciesla DJ, Moore EE, Johnson JL, et al. Multiple organ dysfunction during resuscitation is not postinjury multiple organ failure. Arch Surg. 2004; 139:590–594. [PubMed: 15197083]
15. Lederer JA, Rodrick ML, Mannick JA. The effects of injury on the adaptive immune response. Shock. 1999; 11:153–159. [PubMed: 10188766]
16. Mannick JA, Rodrick ML, Lederer JA. The immunologic response to injury. J Am Coll Surg. 2001; 193:237–244. [PubMed: 11548792]
17. Biberthaler P, Bogner V, Baker HV, et al. Genome-wide monocytic mRNA expression in polytrauma patients for identification of clinical outcome. Shock. 2005; 24:11–19. [PubMed: 15988315]
18. Bogner V, Kirchhoff C, Baker HV, et al. Gene expression profiles are influenced by ISS, MOF, and clinical outcome in multiple injured patients: a genome-wide comparative analysis. Langenbecks Arch Surg. 2007; 392:255–265. [PubMed: 17404753]
19. Committe on Injury Scaling. Abbreviated Injury Scale 1990 Revision. Association of the Advancement of Automovement Medicine; Des Plaines, IL: 2002. (1990)
20. Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc Natl Acad Sci USA. 2001; 98:31–36. [PubMed: 11134512]
21. Simon R, Radmacher MD, Dobbin K, McShane LM. Pitfalls in the use of DNA microarray data for diagnostic and prognostic classification. J Natl Cancer Inst. 2003; 95:14–18. [PubMed: 12509396]
22. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA. 2001; 98:5116–5121. [PubMed: 11309499]
23. MacLennan S, Williamson LM. Risks of fresh frozen plasma and platelets. J Trauma. 2006; 60:S46–S50. [PubMed: 16763481]
24. Rebulla P, Porretti L, Bertolini F, et al. White cell-reduced red cells prepared by filtration: a critical evaluation of current filters and methods for counting residual white cells. Transfusion. 1993; 33:128–133. [PubMed: 8430451]
25. Moore FA, Moore EE, Sauaia A. Blood transfusion. An independent risk factor for postinjury multiple organ failure. Arch Surg. 1997; 132:620–624.
26. Ikuta S, Miki C, Hatada T, et al. Allogenic blood transfusion is an independent risk factor for infective complications after less invasive gastrointestinal surgery. Am J Surg. 2003; 185:188–193. [PubMed: 12620553]
27. Moore EE, Johnson JL, Cheng AM, Masuno T, Banerjee A. Insights from studies of blood substitutes in trauma. Shock. 2005; 24:197–205. [PubMed: 16135956]
28. Ceremuga TE, Yao XL, Alam HB, McCabe JT. Alterations of cullin-5 mRNA levels in the rat central nervous system following hemorrhagic shock. Neurol Res. 2003; 25:211–216. [PubMed: 12635525]
29. Majetschak M, Krehmeier U, Bardenheuer M, et al. Extracellular ubiquitin inhibits the TNF-alpha response to endotoxin in peripheral blood mononuclear cells and regulates endotoxin hyporesponsiveness in critical illness. Blood. 2003; 101:1882–1890. [PubMed: 12406900]

30. Majetschak M, Cohn SM, Obertacke U, Proctor KG. Therapeutic potential of exogenous ubiquitin during resuscitation from severe trauma. J Trauma. 2004; 56:991–999. [PubMed: 15179237]

31. Biberthaler P, Stegmaier J, Mayer V, et al. Initial posttraumatic translocation of NF-kappaB and TNF-alpha mRNA expression in peripheral blood monocytes of trauma patients with multiple injuries: a pilot study. Shock. 2004; 22:527–532. [PubMed: 15545823]

32. Liu SF, Malik AB. NF-kappa B activation as a pathological mechanism of septic shock and inflammation. Am J Physiol Lung Cell Mol Physiol. 2006; 290:L622–L645. [PubMed: 16531564]

33. Endo H, Nito C, Kamada H, Yu F, Chan PH. Akt/GSK3beta survival signaling is involved in acute brain injury after subarachnoid hemorrhage in rats. Stroke. 2006; 37:2140–2146. [PubMed: 16794215]

34. Williams DL, Ozment-Skelton T, Li C. Modulation of the phosphoinositide 3-kinase signaling pathway alters host response to sepsis, inflammation, and ischemia/reperfusion injury. Shock. 2006; 25:432–439. [PubMed: 16680006]

35. Ueki K, Algenstaedt P, Mauvais-Jarvis F, Kahn CR. Positive and negative regulation of phosphoinositide 3-kinase-dependent signaling pathways by three different gene products of the p85alpha regulatory subunit. Mol Cell Biol. 2000; 20:8035–8046. [PubMed: 11027274]

36. Lang R, Hammer M, Mages J. DUSP Meet Immunology: Dual Specificity MAPK Phosphatases in Control of the Inflammatory Response. J Immunol. 2006; 177:7497–7504. [PubMed: 17114416]

37. Dong C, Davis RJ, Flavell RA. MAP kinases in the immune response. Annu Rev Immunol. 2002; 20:55–72. [PubMed: 11861597]

38. Hensler T, Heinemann B, Sauerland S, et al. Immunologic alterations associated with high blood transfusion volume after multiple injury: effects on plasmatic cytokine and cytokine receptor concentrations. Shock. 2003; 20:497–502. [PubMed: 14625472]

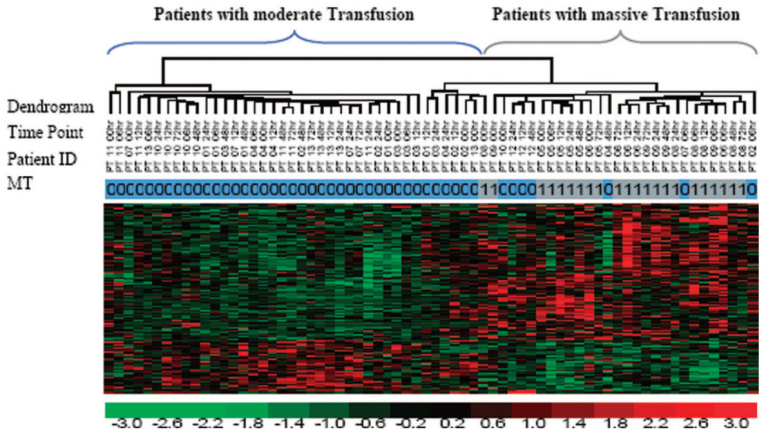

Figure 1.

Supervised analysis of massive RBC transfusion: This graph depicts the hierarchical cluster analysis of the 224 probe sets, which are differentially expressed ($p < 0.001$) in the subjects depending on the amount of administered RBC units. The arrays clustering on the left hand side of the figure belong to patients having received less than 10 RBC units (3,000 mL erythrocyte concentrate, n = 9) during the first 24-hour posttrauma, whereas the cluster on the right hand side consists of arrays from patients who underwent massive blood transfusion (more than 10 RBC units, n = 4).

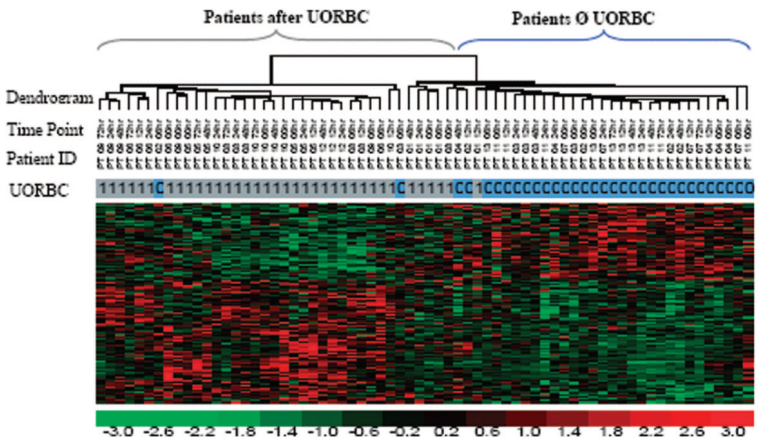

Figure 2.

Supervised analysis of UORBC transfusion. The hierarchical cluster analysis of the 331 probe sets, which are differentially expressed ($p < 0.001$) in the patients who were supplied with UORBC units, when compared with those who exclusively gained crossmatched RBC units. The arrays clustering on the left hand side of the graph belong to patients having received UORBC units (n = 7) during the first 72-hour posttrauma, whereas the cluster on the right hand side consists of arrays from patients who were resuscitated with crossmatched RBC units only (n = 6).

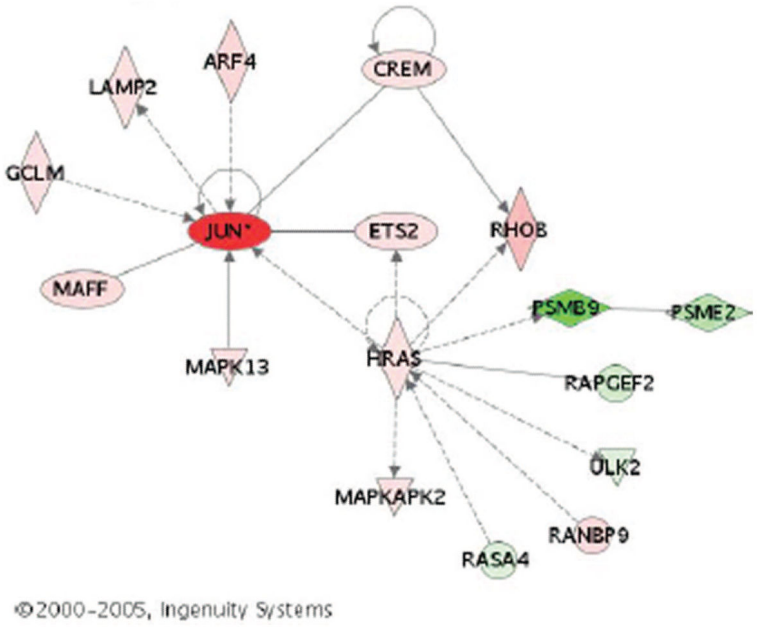

Figure 3.

Pathway analysis of the massive transfusion gene set. A functional network of the pathway analysis of the 224 identified probe sets concerning the criterion “moderate versus massive RBC transfusion.” Interestingly, this network connects elements of the PI3K/AKT pathway and the MAP-Kinase pathway. Red colored gene symbols show an upregulation of gene expression in the patient collective who underwent massive transfusion, and gene symbols in green stand for downregulation in this patient collective. For patients with moderate RBC transfusion, the coloring counts vice versa. The color intensity additionally correlates with the extent of the gene expression change between the two clinical groups. The probability to generate this network by chance alone is 10^{-21}.

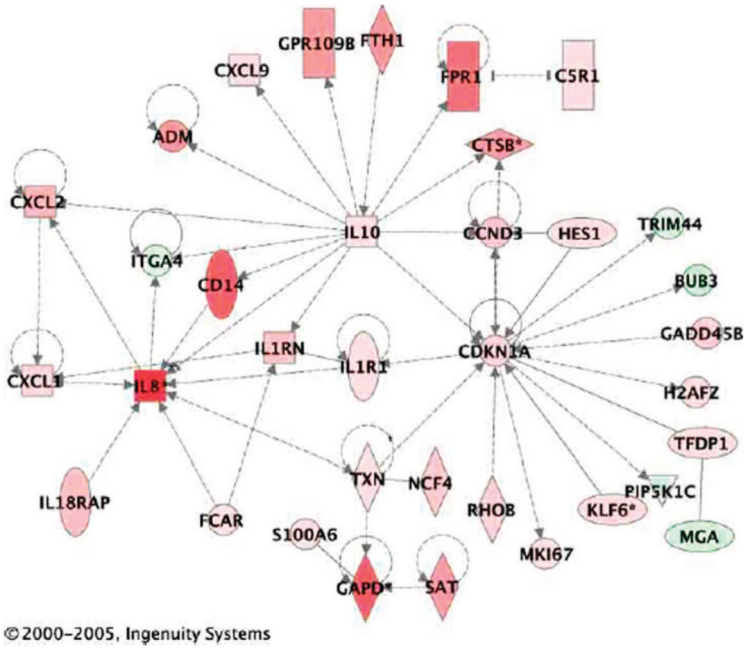

Figure 4.

Pathway analysis of the UORBC transfusion gene set, network 1: the first functional network of the pathway analysis of the 331 identified probe sets concerning the criterion “transfusion of crossmatched versus uncrossmatched RBC units.” The main field of duty of these differentially expressed gene products consists in pro- and antiinflammatory immune system regulation. Red colored gene symbols show an upregulation of gene expression in the patient collective with UORBC unit application, beyond that, color encoding is equally managed as in the precedent analysis. The probability to generate this network by chance alone is 10^{-59}.

TABLE 1

Clinical Baseline Characteristics

Patient ID	Age	Sex	ISS	RBC	FFP	Mass Transfusion	UORBC	Clinical Group	Outcome (at 90 d)
1	53	F	32	8	17	No	Yes	2 and 3	Survived
2	19	M	29	2	6	No	No	2 and 4	Survived
3	48	M	34	10	6	No	No	2 and 4	Survived
4	53	M	19	1	0	No	No	2 and 4	Survived
5	38	M	33	25	22	Yes	Yes	1 and 3	Deceased
6	60	M	50	31	38	Yes	Yes	1 and 3	Deceased
7	36	M	29	0	0	No	No	2 and 4	Survived
8	44	M	57	17	20	Yes	Yes	1 and 3	Survived
9	46	M	34	29	21	Yes	Yes	1 and 3	Survived
10	36	M	54	8	10	No	Yes	2 and 3	Survived
11	54	M	41	3	5	No	No	2 and 4	Survived
12	28	F	75	4	0	No	Yes	2 and 3	Deceased
13	65	M	29	1	0	No	No	2 and 4	Survived

The clinical baseline characteristics of our investigated patients, e.g., age, injury severity, applied RBC and FFP units, and clinical outcome.