Leukodepleted blood components do not remove the potential for long-term transfusion-associated microchimerism in Australian major trauma patients

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Abbreviations: RBC, red blood cell; ISS, injury severity score; SIRS, systemic inflammatory response syndrome; CI, confidence interval; InDel, insertion deletion polymorphisms; LOS, length of stay.

Despite the introduction of leukodepleted blood components, it has been shown that donor leukocyte engraftment (microchimerism) remains a long-term consequence of red blood cell (RBC) transfusion. The incidence of microchimerism may be affected by international disparities in blood processing methods or variations in transfusion practices. This study was conducted to determine the prevalence of microchimerism in Australian trauma patients. A secondary aim was to examine whether any patient complications correlated to the incidence of microchimerism. Australian trauma patients ($n = 86$) who had been transfused with red blood cell (RBC) units between 2000 and 2012 with an injury severity score (ISS) of greater than 15 were recruited. The prevalence of microchimerism was determined using genetic screening with a panel of insertion/deletion biallelic polymorphisms. The mean storage age of the transfused RBC units was 20 ± 8 days and the mean length of stay (LOS) in hospital was 40 ± 39 days. There were no significant associations in this study sample to bias for patient age, gender, number of transfused RBC units or ISS. Nine of the 55 (16.3%) patients transfused with non-leukodepleted blood components displayed an incidence of microchimerism. Of the 31 patients transfused with leukodepleted RBC units, 3 (9.6%) displayed an incidence of microchimerism. Therefore, despite the universal introduction of leukodepleted blood components in Australia, the prevalence of transfusion-associated microchimerism was found to be unchanged. Furthermore, half of the patients exhibiting microchimerism were recorded to have had splenic injury or required splenectomy at the time of transfusion.

Introduction

It has been speculated that transfusion related adverse patient outcomes, such as febrile reactions, transfusion transmitted viruses and blood group antigen alloimmunization are mediated by the presence of viable donor white blood cells (leukocytes) within red blood cell (RBC) units.^{1,2} To prevent these outcomes leukodepletion, which is the removal of donor white blood cells via filtration, was implemented in October 2008 for all Australian manufactured RBC units. The aim of leukodepletion was to reduce the risk of leukocyte related adverse events and this filtration has been successfully associated with reduction in the frequency of nonhemolytic febrile transfusion reactions, human leukocyte antigen (HLA) alloimmunization and risk of transfusion transmitted viruses such as cytomegalovirus, human immunodeficiency virus and hepatitis in most applications.³ However, it has been noted that a leukodepleted RBC unit can contain up

to one million donor leukocytes and still be within specification under the Council of Europe guidelines and clinical studies suggest that RBC transfusion is still associated with undesirable transfusion outcomes.^{4,5}

One such undesirable outcome is termed microchimerism, which is the co-existence of small numbers of genetically disparate cell populations within a single host.⁶ Usually transfused donor cells are recognized by the recipient immune system and are removed within a few days of transfusion. In cases of transfusion-associated microchimerism, donor leukocytes have been detected using sensitive genetic testing across a number of lineages of leukocytes which is suggestive of broad hematopoietic engraftment and proliferation within the recipient.^{7,8}

Microchimerism may be transient or endure for years and may arise after transfusion of a single unit of non-leukodepleted blood component.^{8,9} It has been suggested that the incidence of microchimerism is more common in patients who receive multiple

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blood units or who undergo multiple blood transfusions. The risk may also increase further where there is significant perturbation of the immune response at the time of transfusion, such as in the case of trauma patients.¹⁰⁻¹³ In previous studies, the presence of microchimerism has been reported to persist in 10–15% of major trauma patients and may account for up to 5% of the recipient's circulating hematopoietic cells.^{8,10}

The current knowledge of the long-term clinical consequences and complications for individuals with microchimerism is scarce since long-term studies are difficult to perform. There has been limited new research into the cause and health related outcomes in transfusion-associated microchimerism, however investigations into other settings of microchimerism, such as fetal-maternal microchimerism, have shown an association with a wide array of both chronic immune-related and non-immune diseases.^{14,15} It has been speculated that microchimerism could increase the risk of transfusion-associated graft vs host disease (GvHD). Even though it is a rare complication of allogeneic blood transfusion, in countries such as Japan where there is high ethnic homogeneity of the blood supply, the increased risk of GvHD has led to the routine irradiation of all blood components.¹⁶ Where irradiated blood components are not commonly used, the effect of subtle molecular changes after transfusion could be under reported especially when complications such as multi-organ failure are readily attributable to the original trauma. Furthermore, it has been speculated that alloimmunization from microchimerism could adversely affect both the eligibility for transplantation and the ability to procure compatible blood for transfusion.⁶

It is currently unclear whether the introduction of leukodepleted blood components has affected the frequency of microchimerism, with reports of a reduction in incidence in one study¹⁷ and the frequency apparently unchanged in another study.¹⁰ The mechanism by which donor hematopoietic cells engraft is currently unknown but has been speculated as a mutual tolerance between recipient and donor stem cells.⁸ International disparities in blood collection and manufacturing may affect the incidence of subtle transfusion related outcomes between countries. The incidence of transfusion-associated microchimerism and the effect of leukodepleted blood components within Australian transfusion recipients has not been previously reported. This study aimed to determine whether the introduction of leukodepleted blood components affected the incidence of transfusion-associated microchimerism in Australian trauma patients who received a blood transfusion. A secondary aim of the study was to determine whether any demographic, blood component related or immunologically related complications following transfusion were connected with the prevalence of transfusion-associated microchimerism.

Results

Participant demographics and disposition

Institutional trauma registries identified 360 potential participants who matched the inclusion criteria for injury severity score with associated transfusion of RBC units. Participants

were approached via letter and follow up phone conversation where 58 (16%) could not be contacted by either method. Of the remaining 302 participants, 101 (33%) consented to provide a single blood sample for analysis. Of those who consented, 15 (14.9%) were excluded following analysis of their medical notes due to previous pregnancies or multiple transfusions crossing over the pre-leukodepletion and post-leukodepletion time period. For the participants whose samples were used for analysis (n = 86), 55 received non-leukodepleted RBC units and 31 received leukodepleted RBC units.

Participants were predominantly male (n = 79) with a mean age at the time of trauma as 42 ± 15 years (range 14 – 65). For this study, participants had trauma resulting from motor vehicle accidents (n = 60), falls (n = 10), crush injuries (n = 7), bicycle accidents, stab wounds or burns (n = 9).

Analysis of participant characteristics with relation to leukodepletion status

Table 1 summarizes the findings of the type of trauma with relation to the transfusion of leukodepleted blood components. ANOVA analysis on the type of trauma and leukodepletion status of blood components was not significant (p value = 0.294). Fisher's exact chi square analysis indicated that there was no significant difference between leukodepletion status of blood components and the gender of participants in this study sample (p value = 0.093).

Table 2 summarizes participant characteristics and demographic data in relation to the leukodepletion status of blood components. Independent t-tests were conducted to analyze the significance of each variant in relation to leukodepletion status. The mean age of the participants who received non-leukodepleted RBC units was 42 ± 14 years and for those who received leukodepleted RBC units was 42 ± 17 years which was not significant (**Table 2**). Injury severity scores (ISS), the length of stay (LOS) in hospital and LOS in intensive care (ICU) or the high dependency unit (HDU) was also not significant (**Table 2**). The ICU/HDU LOS was further stratified into short versus long stay, where a short stay was defined as 5 days or less. There was no significant association between leukodepletion status of the transfused blood components and short vs long ICU/HDU stay (p value = 0.262).

The number and type of blood component which were transfused into participants spanning the pre- and post-leukodepletion

Table 1. Cause of trauma stratified by leukodepletion status

Type of accident	Total number of participants	N transfused with leukodepleted RBC units
Motor vehicle accident	54	22
Fall	10	3
Pedestrian	6	3
Crush	7	2
Bicycle	5	0
Stab	1	0
Burn	1	1
Animal related injuries	2	0

Table 2. Participant characteristics and demographics associated with leukodepletion status

Measure	Non-leukodepleted (n = 55)	Leukodepleted (n = 31)	df	t	P-value	95% confidence interval
	Mean $\pm$ SD	Mean $\pm$ SD				
Age (years)	41.9 $\pm$ 13.8	42.2 $\pm$ 17.2	84	0.074	0.941	−6.5, 7.0
Injury Severity Score (ISS) ^a	30 (IQR 21)	29 (IQR 21)	84	0.705	0.483	−3.7, 7.8
Length of stay (days)	39.8 $\pm$ 44.7	40.5 $\pm$ 25	84	0.083	0.934	−16.6, 18
Length of stay ICU/HDU (days)	7.5 $\pm$ 7.5	9.8 $\pm$ 8.7	79	1.259	0.212	−1.3, 5.9
RBC unit transfused	10.8 $\pm$ 8.3	14.8 $\pm$ 9.3	84	2.039	0.053	−0.1, 8
Age of blood (days) ^b	20.8 $\pm$ 8	19.6 $\pm$ 9.2	68	−0.595	0.554	−5.3, 2.9
Platelet units transfused	0.8 $\pm$ 2.3	0.6 $\pm$ 1	84	−0.516	0.608	−1.1, 0.6
FFP units transfused	3.2 $\pm$ 4.5	7.3 $\pm$ 6.9	84	3.364	0.001	1.7, 6.6
Cryoprecipitate units transfused	1.6 $\pm$ 4.2	8.7 $\pm$ 10.7	84	4.36	0.000	3.9, 10.3

^amedian and interquartile range (IQR) shown.

^bage of blood data only available for limited participants (n = 70).

eras was analyzed. It was determined that the mean number of RBC units transfused pre-leukodepletion was 10.8 ± 8.3 (range 2 – 37) and the mean number of RBC units transfused post-leukodepletion was 14.8 ± 9.3 (range 3 – 39), which was not significantly different (Table 2). There was also no significant difference between the number of platelet units transfused into patients in the pre- or post-leukodepletion eras (Table 2). However the difference in the number of fresh frozen plasma (FFP) and cryoprecipitate units transfused in the pre- and post-leukodepletion eras was found to be significant (Table 2).

For participants where complete age of blood data was available (n = 70), it was found that the mean age of RBC units at the time of transfusion for non-leukodepleted RBC units was 20.8 ± 8 days (range 9 – 42) and leukodepleted RBC units was 19.6 ± 9.2 days (range 7– 40) which was not statistically significant (Table 2).

Multivariate logistic regression analysis was conducted to determine if there was any effect causes from participant demographics and number and type of transfused blood components against leukodepletion era. There were no significant associations identified with the exception of the difference in the number of FFP and cryoprecipitate units transfused in the pre-leukodepletion and post-leukodepletion eras.

Analysis of microchimerism status

The presence of microchimerism was determined from genomic DNA isolated from the buffy coat provided by each participant. The method of microchimerism determination is described in detail in the methods section. However in brief, 12 unique biallelic insertion/deletion (InDel) regions of the genome were amplified. Each participant had an allogeneic InDel pattern which amplified early in the polymerase chain reaction. Microchimerism was classified to be present if there was amplification of 1 to 3 unique minor allele types with cycle threshold 8 to 10 cycles later than the allogeneic InDel sequence pattern.

When performed on this study cohort InDel analysis indicated 12 participants displayed amplification patterns consistent with microchimerism. Of these 12 microchimeric participants, 9 had received non-leukodepleted RBC units and 3 had been transfused with leukodepleted RBC units. Fisher's exact chi square

analysis indicated that the incidence of microchimerism in participants transfused with non-leukodepleted RBC units vs. those transfused with leukodepleted RBC units was not significant (p value = 0.524).

Table 3 summarizes the type of trauma sustained by each participant with relation to the incidence of microchimerism. ANOVA calculations indicated there was no significant difference between the type of trauma and the incidence of microchimerism (p value = 0.381). Fisher's exact chi square analysis also indicated no significant associations between the gender of participants and the incidence of microchimerism (p value = 1.000).

Table 4 summarizes participant characteristics and demographic data in relation to the incidence of microchimerism. Independent t-tests were conducted to analyze the significance of each variant in relation to the incidence of microchimerism. The mean age of the participants was not significantly different between those who had no microchimerism (42.2 ± 15 years) and those who had microchimerism (41 ± 16 years) (Table 4). The ISS, LOS or ICU/HDU LOS between non-microchimeric and microchimeric individuals was also not significant (Table 4). Short versus long ICU/HDU stays were also analyzed and was found not to be significantly associated with the incidence of microchimerism (p value = 0.762). There was also no significant correlation between the number of RBC units transfused and the incidence of microchimerism (Table 4). There was no significant relationship between the incidence of microchimerism and the number of platelet, FFP and cryoprecipitate units transfused (Table 4).

Table 3. Cause of trauma stratified by the incidence of microchimerism

Type of accident	Total number of participants	N with microchimerism
Motor vehicle accident	54	6
Fall	10	1
Pedestrian	10	1
Crush	7	2
Bicycle	5	1
Stab	1	1
Burn	1	0
Animal related injuries	2	0

Table 4. Participant characteristics and demographics associated with the incidence of microchimerism

Measure	No microchimerism (n = 74)	Microchimerism (n = 12)	df	t	P-value	95% confidence interval
	Mean $\pm$ SD	Mean $\pm$ SD				
Age (years)	42.2 $\pm$ 15	41 $\pm$ 16	84	0.226	0.822	−8.3, 10.4
Injury Severity Score (ISS) ^a	33 (IQR 20)	25.5 (IQR 27)	84	0.808	0.421	−4.7, 11.1
Length of stay (days)	38.9 $\pm$ 32.3	47 $\pm$ 67.1	84	−0.673	0.503	−32.1, 15.8
Length of stay ICU/HDU (days)	8.4 $\pm$ 8	8.2 $\pm$ 8.1	79	0.107	0.915	−4.7, 5.3
RBC units transfused	12.6 $\pm$ 9.3	9.9 $\pm$ 4.7	84	0.994	0.323	−2.7, 8.2
Age of blood (days) ^b	20.1 $\pm$ 8.9	21.2 $\pm$ 6	68	−0.361	0.719	−6.9, 4.8
Platelet units transfused	0.78 $\pm$ 2	0.58 $\pm$ 1.2	84	0.333	0.74	−1, 1.4
FFP units transfused	4.9 $\pm$ 6	3.4 $\pm$ 4.2	84	0.808	0.422	−2.1, 5
Cryoprecipitate units transfused	4.6 $\pm$ 8.4	1.7 $\pm$ 3.9	84	1.186	0.239	−2, 7.9

^amedian and interquartile range (IQR) shown.

^bage of blood data only available for limited participants (n = 70).

For participants where complete age of blood data was available (n = 70), it was found that the mean age of RBC units at the time of transfusion for participants without microchimerism was 20.1 $\pm$ 8.9 days (range 7 – 42) and with microchimerism was 21.2 $\pm$ 6 days (range 14– 32) which was not statistically significant (Table 4).

Analysis of blood components given early during admission vs. components given over the length of admission was also conducted. For this study, blood given early was classified as either a massive transfusion of blood components ($\geq$ 5 units of red blood cells within 4 hours or $\geq$ 10 units of red blood cells within 24 hours) or where a participant was transfused with their total number of blood components (including FFP, cryoprecipitate and platelet) within 24 hours of hospital admission. Fisher's exact chi square analysis of blood early versus blood late in relation to the incidence of microchimerism was found not to be significant (p value = 0.534).

Multivariate logistic regression analysis was also conducted on all of the noted demographic and blood component characteristics to determine if there was any combined effect of these factors

on the incidence of microchimerism. When analyzed there was no significant associations found within this study sample.

Fisher's exact chi square tests were then conducted to determine the association of the incidence of microchimerism and the following immunologically related patient complications; fever following blood transfusion, pneumonia (ventilator or hospital-acquired), systemic inflammatory response syndrome (SIRS) or wound infections. It was found that there were no significant associations between these noted clinical complications and the development of microchimerism (Table 5). Fisher's exact chi square analysis of comorbidities including obesity, hepatitis, asthma, cardiac/blood pressure complications, diabetes, multiple sclerosis or appendectomies were also not found to be significantly associated with the incidence of microchimerism (Table 5).

However, of particular note, 16 of the 86 study participants had spleen injury at the time of admission. For this study, spleen injury was defined as splenic laceration or tear, or damage requiring a splenectomy. Ten of these participants, of which 9 required splenectomy, did not have microchimerism. The remaining 6

Table 5. Analysis of immunological outcomes and comorbidities and association with the incidence of microchimerism^a

Reported clinical indication/comorbidity	No microchimerism (n = 74)		Microchimerism (n = 12)		Fisher's exact test p-value (2-tailed)
	n	%	n	%	
Febrile	39	53	6	50	—
Pneumonia – ventilator or hospital acquired	12	16	5	42	0.055
SIRS	13	17	3	25	0.689
Infection (bacterial or fungal)	33	45	3	25	0.344
Rash	0	0	1	8	0.140
Obesity	4	5	0	0	—
Hepatitis	1	1	0	0	—
Asthma	6	8	1	8	—
Multiple Sclerosis	1	1	0	0	—
Cardiac	5	7	2	17	0.251
Diabetes	3	4	0	0	—
Marfan Syndrome	2	3	0	0	—
Bipolar Syndrome	1	1	0	0	—
Appendectomy	1	1	1	8	0.261

^amore than one indication may occur per participant; effect size was not large enough for effective use of statistical models.

spleen injured participants displayed microchimerism and the spleen injury was attributed to spleen tears or lacerations ($n = 4$) not splenectomy. Fisher's exact chi square analysis indicated a significant association between spleen injury and incidence of microchimerism with p -value = 0.007 (odds ratio 6.5 and CI 1.7, 24.2). With the removal of splenectomies from the analysis an association between splenic lacerations or tears and incidence of microchimerism was still evident (p value = 0.001, odds ratio 43.3, CI 4.2, 452.3).

Multivariate logistic regression modeling was conducted to analyze all of the noted clinical indicators and comorbidities against the incidence of microchimerism with the aim of determining if there was continued sensitivity of the spleen injury finding. The effect sizes for most comorbidities were mostly insufficient but spleen injury was still found to be significant.

Discussion

International studies on blood transfusion related outcomes may be difficult to correlate due to differences in blood manufacturing processes and transfusion practices. Limited research into long-term transfusion related outcomes, such as transfusion-associated microchimerism, has not previously been conducted particularly in the context of Australian transfusion recipients. This retrospective study analyzed a sample of Australian major trauma patients who were transfused with red blood cell units before and after the introduction of leukodepletion filtration. Leukodepletion was introduced to reduce the potential of donor leukocytes causing viral transmission, febrile episodes and HLA alloimmunization and has in the most part been successful in removing these adverse transfusion related outcomes.^{1,18} However, the incidence of microchimerism was not significantly impacted by the introduction of leukodepleted RBC units in this study sample. This continued effect despite the use of leukodepleted blood products along with the prevalence of 9% is similar to previous findings from US-based studies.^{10,12,19}

Furthermore in this cohort, microchimerism was detected in patients transfused up to 12 years prior to this analysis. To our knowledge this is the second group of retrospective analyses to report long-term microchimerism spanning over a decade from the date of blood transfusion, with previous work in war veterans displaying microchimerism tracing back 60 years.¹⁹

The mechanisms enabling long-term donor cell survival are unknown but it has been shown that trauma is necessary but not sufficient to result in microchimerism. It is likely that transient immune disarray following trauma allow for donor cells to evade the host immune system but it is unclear why they persist in some individuals after the host system is restored and whether they are functional. It is proposed that the cells which cause microchimerism are multilineage hematopoietic cells but no specific cell type has been proven definitively to cause this phenomenon.⁸ Persistence of tolerated donor cells may also occur from long-term survival of differentiated cells and does not appear to

be leukocyte dose dependant as the number of RBC units transfused differs between individuals.

There is speculation about the age of blood at the time of transfusion leading to an increased risk of transfusion-associated microchimerism.^{11,20} In this study cohort there were no significant associations between the median age of blood transfused between microchimeric and non-microchimeric individuals. There was also no significant association between the minimum storage time (no microchimerism 12.8 ± 9.5 days, microchimerism 13.7 ± 7.2 days, p value 0.773) and maximum storage time (no microchimerism 30.3 ± 9.3 days, microchimerism 33.6 ± 6.2 days, p value 0.282). Microchimerism was detected in individuals despite being given older units which is similar to previous studies.²⁰ There was also no significant associations whether the blood was given early (within 5 days) during the hospital admission or distributed throughout the length of stay in hospital.

There is the potential for genetic predisposition to microchimerism, although no specifically studied haplotype or polymorphism has been predictive of transfusion-associated microchimerism in trauma patients thus far.^{8,21}

Immunological complications in patients usually increase their clinical outcomes or length of stay in hospital, ICU or HDU. In this study cohort no significant associations were established for demographics (age or gender) or trauma related clinical indicators (ISS, type of trauma, LOS, ICU/HDU LOS) whether the data was stratified by leukodepletion era or incidence of microchimerism. The exception to this was the difference in the number of transfused FFP and cryoprecipitate units in the pre- and post-leukodepletion eras. It is most likely that this difference was apparent since patient blood management methods were altered across Australia under revised critical bleeding massive transfusion guidelines produced by the National Blood Authority in 2011 (<http://www.blood.gov.au>). These guidelines affected the use of each blood component in critical bleeding scenarios and appear significant as this introduction generally overlapped with use of leukodepleted RBC units.

The number of FFP and cryoprecipitate units transfused was not significant when the data was stratified for the incidence of microchimerism. Furthermore even though platelets are inherently immunogenic, the number of transfused platelet units between microchimeric and non-microchimeric individuals was not significant. Additionally in all cases where platelets were transfused into microchimeric individuals the components had been irradiated prior to use. Therefore it is most likely that the red blood cell components are the source of surviving donor leukocytes and the immunogenic nature of platelets has a minimal impact on the incidence of microchimerism.

This study was limited to participants who had good long-term outcomes following trauma and transfusion. It remains unknown whether the subtle molecular changes which occur in transfusion-associated microchimerism are correlated with any long-term health potential or whether these cells are just tolerated as a consequence of transfusion. There is the potential for surviving donor white cells to have a more immediate impact in immunosuppressed recipients and be the cause of multi-organ failure

or other immunologically related complications. This limitation of not being able to include patients who had died from their injuries or had other poor trauma related outcomes restricts the gathering of this type of clinical consequence data.

This study was limited in sample size; nevertheless study participation rates were similar to previous studies.^{10,12,22} The skew toward male participants was exacerbated by the exclusion of some female participants from the analysis due to pregnancies. Removal of previously pregnant females from the study was required to prevent any confusion with the detection of naturally occurring fetal/maternal microchimerism.²³

There were also limitations in the indications of possible immune suppression since these were sought from each participant's medical records and were only indicators that were visible to the hospital staff or those which were specifically tested for by laboratories. Even though no associations were found in this study between the incidence of microchimerism and fever following transfusion, wound infections, SIRS, comorbidities and other respiratory complications this is not a comprehensive analysis of the clinical or biological status of the patient at the time of transfusion. Further studies to analyze biological changes following transfusion would be required and have been conducted in cancer patients where microchimerism in patients who received non-leukodepleted RBC units showed specific immunomodulatory changes.¹⁷

In this study there was a significant association between spleen injury and microchimerism. There is the potential that the individuals with spleen injuries may have a differing cytokine profile which favors microchimerism. The finding of spleen lacerations/tears with microchimerism is unique where previously no associations were identified although only splenectomy rates were reported.¹³ Injury to the spleen could provide clues for mechanisms by which microchimerism might establish within individuals. The spleen is an important organ for filtering abnormal cells and for producing antibodies and lymphocytes. If ruptured at the time of transfusion there may be sufficient immunological disarray to enable donor leukocytes to evade the immune system and engraft.

It is important to note that many of the participants in this study with spleen injury and microchimerism were within the pre-leukodepleted blood component group. Thus there is a requirement to study more patients who have had a spleen injury and received leukodepleted RBC units before any inferences can be reached. However, this association has the potential to provide a clinical indicator to identify which patients are most likely to develop microchimerism prior to red blood cell transfusion.

The continued incidence of donor cell survival in major trauma patients post leukodepletion is of interest since the long-term clinical consequences of donor cell survival in transfusion recipients remains unclear. It is unknown if there are subtle adverse or beneficial effects from the presence of donor cells. Microchimerism may be harnessed to predict transplantation outcomes or equally these cells may remain anergic and this phenomenon may just be a remnant of the sustained physical trauma coupled with the inherent trauma of transfusion.

Materials and Methods

Selection of donors

The study was approved by the Western Sydney Local Health District Human Research Ethics Committee and the Australian Red Cross Blood Service Human Research Ethics Committee with governance granted at other New South Wales based hospital sites under the harmonization of multi-center ethical review (HoMER) process (National Health and Medical Research Council, Australia).

Trauma patients were identified retrospectively from the institutional trauma registries of participating trauma centers. Potential participants were approached by the study team via an invitation letter and a follow-up phone call and enrolled after providing written informed consent. Participants had been transfused in the period from September 2000 until December 2012. Since leukodepletion was universally introduced for RBC units in Australia from the first of October 2008, patients who had been transfused up to 6 weeks following this date were excluded from the pool to exclude the possibility that patients were transfused with mixed leukodepleted and non-leukodepleted blood components.

Eligible patients were identified with ISS greater than 15, who had received transfusion of RBC units and were able to provide written informed consent at the time of the study. Patients were excluded for current or prior pregnancy (including any pregnancies which did not go to term), being under the age of 18 when approached for consent, imprisonment at the time of recruitment or having a history of blood transfusion since those transfusions may have occurred with a mixture of leukodepleted and non-leukodepleted RBC components.

Blood collection

Participants provided a single non-fasting blood specimen in a 6mL ethylenediaminetetraacetate (EDTA)-anticoagulant collection tube (Cat # 367876; BD biosciences) using standard phlebotomy techniques at commercial pathology collection centers. Specimens were shipped to the Blood Service research laboratories for processing within 36 hours. The buffy coat layer containing all nucleated cell populations was obtained by centrifugation of the blood sample at $1000 \times g$ for 10 minutes and were stored in 2 aliquots frozen at -80°C .

Real time PCR

Genomic DNA was extracted using ReliaPrep™ blood gDNA miniprep extraction kit (Cat # A5082; Promega) from a thawed buffy coat aliquot and analyzed for quality and concentration using a Nanodrop spectrophotometer (NanoDrop 2000; Thermo Fisher scientific).

Microchimerism was detected using previously characterized phenotypically silent biallelic insertion or deletion regions spanning 9 different chromosomes. The twelve biallelic markers used to detect these insertion/deletion (InDel) regions were SO-1, SO-2, SO-3, SO-4, SO-4B, SO-6, SO-7, SO-7B, SO-8, SO-8B, SO-9, and SO-11 with exact amplification primer sequences

as previously published.²⁴ Successful use of InDel amplification to detect microchimerism in blood transfusion recipients and hematopoietic stem cell replacement patients has been shown previously.^{12,24,25} GAPDH primers were used as positive amplification controls [F 5' – GGACTGAGGCTCCACCTTT – 3' and R 5' –GCATGGACTGTGGTCTGCAA – 3'].

InDel sequences show a high degree of heterozygosity within the general population, therefore each individual will display a unique pattern of InDel sequence amplification. Detection of genetically distinct cell populations occurs when 2 distinct populations of InDel sequences are amplified within a single reaction. Each population will also be separated by a number of cycle thresholds during PCR which indicates a major and minor allelic population. When the InDel sequences amplify between cycles 19 and 24, this is the major (allogeneic) population. The minor (microchimeric) population amplifies later between PCR cycle number 28 and 32.

To evaluate the lower limit of reliable detection for InDel amplification, genomic DNA was isolated from 2 healthy volunteers. InDel sequences were amplified from each volunteer via real-time PCR to determine their allogeneic InDel amplification pattern. A fixed concentration of genomic DNA from volunteer A (100 ng) was then spiked with a serially diluted concentration (100% to 0.001%) of genomic DNA from volunteer B. It was determined that the sensitivity of reliable InDel amplification was 0.01% which is consistent with previously published assay reports.^{24,25}

To evaluate each participant sample, triplicate PCR reactions were conducted using 50 ng of total genomic DNA template in a 20µL PCR volume consisting of 1× AmpliTaq Gold amplification buffer (Cat # N8080249; Applied Biosystems); 4.5 mM MgCl₂ (Cat # AB-0359; Applied Biosystems); 200 nM each dNTP (Cat # BIO-39025; Boline); 1.25µM Syto9 (Cat # S-3854; Life Technologies); 1 U AmpliTaq Gold DNA polymerase (Cat # N8080249; Applied Biosystems) and 500 nM of each primer (Geneworks). Real-time PCR was conducted using Rotor-Gene Q (Qiagen) with cycle conditions of 10 minutes at 95°C followed by 40 cycles of 30 seconds at 95°C, 15 seconds at 65°C, and 10 seconds at 72°C. Analysis was conducted using Rotor-Gene Q software version 2.2.3 (Qiagen). Quantitation analysis was used with fluorescence change threshold set over 30%.

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Medical and Blood Donation Record analysis

Medical records were viewed for each participant from the date of admission to analyze details of the sustained injuries, identify transfused blood component details, note any comorbidities at the time of admission and note any immunologically related complications following transfusion including systemic inflammatory response syndrome (SIRS), fever, infections or pneumonia. Identifying information for each transfused blood component was used to determine the age of blood at the time of transfusion, irradiation status and other related information to be extracted from blood donation records held within the Australian Red Cross Blood Service national blood management system.

Statistical Analysis and Calculations

Data were analyzed using IBM SPSS statistics 19 (IBM, Armonk, NY). Analysis was performed using Fisher's exact chi-square analysis, independent t-tests, multivariate regression modeling or ANOVA where stated. Significance was given where p-values were greater than 0.05 with 95% confidence interval (CI). Summary measures of continuous variables are shown as the mean ± standard deviation unless otherwise indicated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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