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Red cell alloimmunisation in patients with different types of infections

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Summary

Red cell alloantigen exposure can cause alloantibody-associated morbidity. Murine models have suggested that inflammation modulates red cell alloimmunisation. This study quantifies alloimmunisation risks during infectious episodes in humans. We performed a multicentre case–control study within a source population of patients receiving their first and subsequent red cell transfusions during an 8-year follow-up period. Patients developing a first transfusion-induced red cell alloantibody (N = 505) were each compared with two similarly exposed, but non-alloimmunised controls (N = 1010) during a 5-week 'alloimmunisation risk period' using multivariate logistic regression analysis. Transfusions during 'severe' bacterial (tissue-invasive) infections were associated with increased risks of alloantibody development [adjusted relative risk (RR) 1·34, 95% confidence interval (95% CI) 0·97–1·85], especially when these infections were accompanied with long-standing fever (RR 3·06, 95% CI 1·57–5·96). Disseminated viral disorders demonstrated a trend towards increased risks (RR 2·41, 95% CI 0·89–6·53), in apparent contrast to a possible protection associated with Gram-negative bacteraemia (RR 0·58, 95% CI 0·13–1·14). 'Simple' bacterial infections, Gram-positive bacteraemia, fungal infections, maximum C-reactive protein values and leucocytosis were not associated with red cell alloimmunisation. These findings are consistent with murine models. Confirmatory research is needed before patients likely to develop alloantibodies may be identified based on their infectious conditions at time of transfusion.

Keywords: red blood cell alloimmunisation, blood transfusion, infections, inflammation, humans.

Red cell alloimmunisation challenges the provision of compatible donor blood and, most importantly, might induce severe haemolytic transfusion reactions (Serious Hazards Of Transfusion [SHOT], 2014; Transfusion and Transplantation Reactions In Patients [TRIP], 2014). Consequently, some selected patients receive extensively matched blood (Dutch Institute for Healthcare Improvement [CBO], 2011; United Kingdom Blood Services, 2013). Despite the effectiveness of these risk-based matching practices (Vichinsky *et al*, 2001; Lasalle-Williams *et al*, 2011; Schonewille *et al*, 2015), non-selected patients do experience alloimmunisation-mediated complications (Harvey *et al*, 2014; TRIP, 2014; SHOT, 2015) warranting consideration of additional risk factors.

In addition to the chance of encountering a high immunogenic non-self antigen (Evers *et al*, 2016), clinical conditions affecting the recipient's immune response

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probably modulate alloimmunisation. Identification of such factors might enable allocating extensively matched blood principally to high-risk patients.

Experimentally induced inflammation has consistently been demonstrated as a major determinant of red cell alloimmunisation in mice (Hendrickson *et al*, 2006, 2007; Hendrickson, 2008; Smith *et al*, 2012). In line with this, pro-inflammatory conditions related to sickle cell disease as well as febrile reactions to donor platelets were shown to enhance alloimmunisation in humans (Yazer *et al*, 2009; Fasano *et al*, 2015). Apart from one case report (Hata *et al*, 2013) to the best of our knowledge, the influence of infection-associated inflammation on red cell alloimmunisation in humans has not been reported.

In this nested case–control study, we quantified relative alloimmunisation risks for patients receiving red cell units during an infectious episode, according to the type of infection, its intensity, and the patient's inflammatory response to it.

Patients and methods

Study design and setting

We performed a nested case–control study within a source population of previously non-transfused and non-alloimmunised patients in three university hospitals and three reference hospitals in the Netherlands. Using this design, we compared patients who developed red cell alloantibodies following transfusion with non-alloimmunised controls on the basis of supposed causal attributes, including various types of infections. Details on the source population, including its eligibility criteria, and our case–control study design have been previously published (Zalpuri *et al*, 2012a; Evers *et al*, 2016).

To summarize, patients were eligible if they received their first red cell transfusion during the study period in one of the participating hospitals, provided this transfusion was preceded by a negative antibody screen and followed by an antibody screen, hereby permitting evaluation of alloantibody development. The study period per hospital depended on electronic availability of the necessary data between 1 January 2005 and 31 December 2013 (for details, see Data S1). All red cell units were prepared by buffy-coat depletion of whole blood donations, subsequently filtered through a leucocyte depletion filter, and stored in saline, adenine, glucose, and mannitol (SAGM) for a maximum of 35 days (CBO, 2011).

A patient was defined as a case upon developing a first, transfusion-induced red cell alloantibody directed against one of the following antigens: c, C, e, E, K, C^w, Fy^a, Fy^b, Jk^a, Jk^b, Lu^a, Lu^b, M, N, S or s. Anti-D immunised patients were not taken into consideration because we were unable to discriminate whether anti-D was caused by unmatched transfusions, or (mainly regarding fertile women) was due to recent anti-D administration in the context of a D-positive pregnancy or transfusion. Patients who formed antibodies, yet either lacked exposure to a (documented or assumed) antigen-positive red cell unit or expressed the antigen themselves (i.e. auto-immunised patients) were deemed ineligible. In addition, alloimmunised patients were excluded if their first-time alloantibody positive screen occurred within 7 days of the first mismatched transfusion, as these were more likely to represent boosting of earlier primary immunisations. By consulting the nationwide alloimmunisation registry (http://www.sanquin.nl/producten-diensten/diagnostiek/trix), we additionally excluded patients previously diagnosed with alloimmunisation in other hospitals. Considering the above-mentioned criteria, we specifically aimed to exclude previously alloimmunised patients, including pregnancy-induced immunisations in women. Finally, haemoglobinopathy patients and infants below 6 months of age were not included.

Each eligible case was matched to two randomly selected non-alloimmunised control patients based on the hospital and on the (lifetime) number of red cell transfusions received at the time of alloimmunisation. This 'incidence-density sampling strategy' ensured that controls were exposed to at least the same amount of transfusions as their matched cases and thus formed a representative sample of the source population (Rothman, 2007).

For all cases, we assumed that the last antigen-mismatched transfusion (the '*Nth*' or implicated transfusion) preceding the first positive screen most likely elicited alloimmunisation. If this last mismatched transfusion could not be identified due to incomplete typing of donor units, we assumed the last non-tested unit preceding the first positive screen by at least 7 days to have elicited alloimmunisation. An 'alloimmunisation risk period' was then constructed, stretching from 30 days before up to 7 days after this implicated *Nth* transfusion. A similar risk period around the *Nth* transfusion was determined for the matched controls. The implicated transfusion and its alloimmunisation risk period are illustrated in Fig 1.

For all cases and controls, we recorded various clinical conditions during the alloimmunisation risk period.

The study protocol was approved by the Ethical Review Board at the Leiden University Medical Centre in Leiden and by the local board of each participating centre.

First-formed red cell alloantibodies

Patients in the Netherlands are routinely screened for red cell alloantibodies at a maximum of 72 h prior to red cell transfusion. According to the Dutch transfusion guideline (CBO, 2011), commercially available 3-cell screening panels are required to be homozygous positive for D, C, c, E, e, K, Fy^a, Fy^b, Jk^a, Jk^b, M, S and s. The presence of the K antigen in its heterozygous form is a minimum requirement. The presence of C^w, Lu^a, Wr^a, and Kp^a is not mandatory on commercially available screening cells (CBO, 2011). Antibody screening involves a three-cell panel using an indirect antiglobulin test [column agglutination technology from either BioRad (Cressier, Switzerland), or Ortho Clinical Diagnostics (Raritan, NJ, USA)]. If positive, screening is followed by subsequent

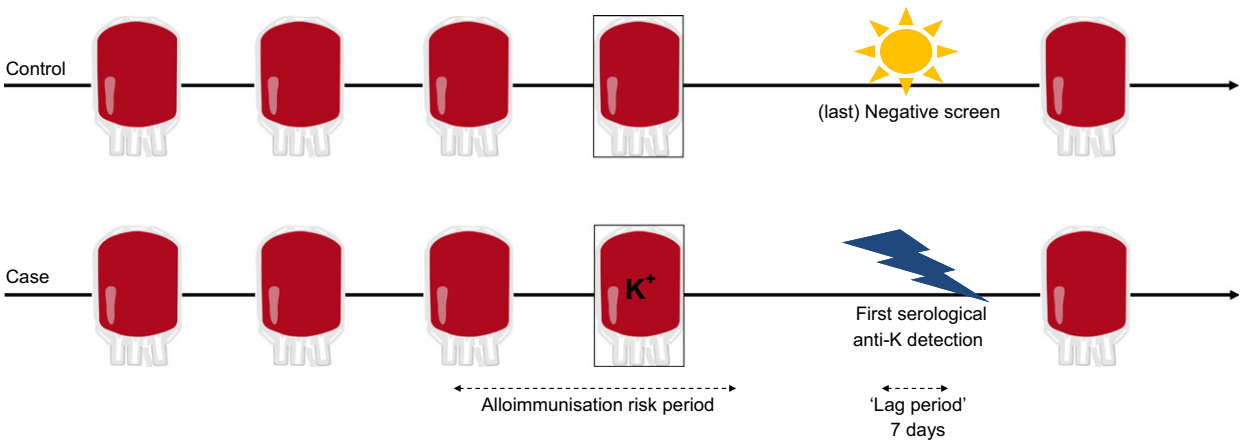

Fig 1. The implicated transfusion and alloimmunisation risk period. The last antigen-mismatched transfusion preceding the first serological detection of an antibody was defined as the 'implicated (or *Nth*) transfusion' since this transfusion was the most likely to influenc alloimmunisation. To exclude possible boosting events, this implicated transfusion was required to precede the first positive screen by at least 7 days (i.e. lag period). An alloimmunisation risk period was then constructed starting 30 days before and finishing 7 days after this implicated transfusion. Controls received at least the same number of red cell units as their matched case. A similar alloimmunisation risk period around the *Nth* matched transfusion was constructed.

antibody identification by an 11-cell panel using the same technique.

Data acquisition

We gathered routinely stored data on red cell transfusion dates, dates and results of antibody screens (including antibody specificity), patients' date of birth, sex and leucocyte counts from the hospitals' electronic laboratory information systems. In addition, we examined the medical charts of all cases and controls for the presence of various potential clinical risk variables during the alloimmunisation risk period, including dates of infection, the causative microorganisms, dates of fever (temperature ≥38·5°C), leucocyte counts, and C-reactive protein (CRP) values.

Bacterial infections comprised tissue-invasive infections (i.e. involving an anatomic site location) and bacteraemia (i.e. involving positive blood cultures).

Tissue-invasive bacterial infections were considered present when confirmed by either a positive blood or tissue culture, or when a suspected clinical infectious phenotype was supported by an overtly disease-specific radiographic anomaly e.g. a clear lobar consolidation on a chest x-ray in a patient with fever and cough. We categorized these infections into 'mild' or 'severe' according to their expected degree of systemic inflammation. Mild tissue-invasive bacterial infections included: routine (tip) cultures from central catheters, catheter-induced phlebitis, lower urinary tract infections, bacterial enteritis, skin and superficial wound infections, and upper respiratory tract infections. 'Severe' tissue-invasive bacterial infections included: abscesses, intra-abdominal infections including spontaneously or secondarily infected abdominal fluid collections, arthritis, bursitis, myositis, fasciitis, infected haematoma, bacterial meningitis, deep wound or skin infections, endocarditis, mediastinitis, pericarditis, infected foreign material, lower respiratory tract infections, osteomyelitis, spondylodiscitis and upper urinary tract infections.

Bacteraemia were categorized according to their Gram-positive or Gram-negative causative microorganism.

For the qualification of a viral infection, a positive polymerase chain reaction (PCR) test demonstrating the replication of viral RNA or DNA was needed or, in case a PCR test was not performed, the clinical condition needed to be clearly virally induced, e.g. herpes labialis. Viraemia and disseminated viral zoster infections were defined as 'disseminated viral infections', in contrast to 'local viral infections', which were restricted to one anatomic site location.

Statistical analyses

The associations of various infections with the development of red cell alloimmunisation were evaluated using logistic regression analyses. For crude relative risk (RR) calculations, we conditioned on the matched variables, i.e. hospital and cumulative number of red cell units received.

For multivariate analyses, we also conditioned on measured confounders, taking into account that a confounder meets the prerequisites of being associated with the exposure (i.e. infections) in the source population, is (a marker for) a causal risk factor of the outcome (i.e. alloimmunisation) and is not in the causal pathway between the exposure and the outcome (Hernan *et al*, 2002; Middelburg *et al*, 2014). Consequently, we used the following strategy. First, we identified a subset of covariates to be confounders of a given determinant based on their observed association with the determinant within the source population (i.e. the non-alloimmunised controls). Such an association was defined as

a ≥3% difference in covariate presence between controls exposed and controls not exposed to the determinant. Covariates in the causal pathway between the determinant and the outcome were not considered as confounders (Hernan *et al*, 2002). Second, to be able to accurately control for confounders with low prevalences, we estimated a probability score for each determinant using logistic regression with the potential confounders as predictors (Brookhart *et al*, 2013). Third, to minimize bias due to missing data on the confounders, we used multiple imputation. Details on the used imputation model can be found in the Data S1 and Table SI. Finally, we evaluated the association between various types of infections and red cell alloimmunisation by subsequently entering the corresponding probability scores into the logistic regression model with alloimmunisation as the outcome and conditioning on the matched variables.

We next assessed the association of level of CRP values and leucocytosis as possible markers of inflammation with red cell alloimmunisation. Leucocytosis was categorized as maximum measured leucocyte counts of 10–15, 15–20, 20–30 and $>30 \times 10^9$/l, and referenced to normal counts ($4–10 \times 10^9$/l). Maximum measured CRP values were categorized as 30–100, 100–200, 200–300 and >300 mg/l, and referenced to values ≤30 mg/l. Missing CRP and leucocyte values were multiply imputed using the same strategy as described above. While the likelihood that an increased inflammatory parameter has been recorded at least once increases with the number of measurements and thus with the duration of hospitalization, we repeated these analyses limited to parameters measured within the week following the implicated transfusion. As elevated CRP levels and leucocytosis reflect various clinical conditions preventing causal inferences, they are presented here only as unadjusted RRs.

As anti-E, anti-C^w, anti-Le^a, anti-Le^b, anti-Lu^a, and anti-M can also form 'naturally' (e.g. directly in response to microbial epitope exposure (Reid *et al*, 2004), we evaluated a possible association between the presence of these antibodies and various types of infections using Pearson's chi-square test. *P*-values <0·05 were considered to be statistically significant.

As we used an incidence-density sampling procedure to select controls (Rothman, 2007), we interpreted and present all odds ratios as RRs with 95% confidence intervals (95% CI).

Sensitivity analyses

For some patients, the presence or absence of a certain type of infection could not be determined. These patients were left out of the corresponding analysis. Regarding severe bacterial infections, we performed a sensitivity analysis in which these patients were alternately assigned to exposure and non-exposure of this infection.

For patients with a suspected lower respiratory infection without conclusive or available cultures, we considered this infection to be due to a bacterial microorganism. Although viral or (rarely) fungal pathogens may cause pneumonia, bacterial microorganisms are the most common cause in Dutch hospitalised patients, with *Streptococcus Pneumoniae* and *Haemophilus Influenzae* alone representing 30–75% of causative pathogens (Wiersinga *et al*, 2012).

Finally, as contaminated blood cultures positive for coagulase-negative staphylococci (CNS) might dilute an existing effect of Gram-positive bacteraemia, we compared RRs for all Gram-positive bacteraemia with those for non-CNS Gram-positive bacteraemia.

Results

Among 54 347 newly-transfused patients, 24 063 were considered eligible (Fig S1) of which 505 patients (2·1%) formed red-cell alloantibodies. Thirty-seven of these alloimmunised patients (7·3%) only received units of which the cognate antigen was unknown. For these, we assumed the last non-tested unit preceding the first positive screen to have elicited alloimmunisation.

General and clinical characteristics of the 505 cases and their 1010 matched controls during the alloimmunisation risk period are presented in Table I.

Infections during the alloimmunisation risk period

Among all cases and controls, 473 patients were diagnosed with at least one infection during the alloimmunisation risk period. Of these, 417 suffered from bacterial infections, 53 from viral infections and 56 from fungal infections (Table II).

For 222 of 269 patients (82·5%) diagnosed with a severe tissue-invasive bacterial infection, the causal microorganism was identified by culture. For three of 53 virally-infected patients, no PCR test was performed during the alloimmunisation risk period. These patients were nevertheless included based on their clinical condition: one patient receiving an allogeneic stem cell transplantation with an outbreak of varicella zoster, one patient receiving chemotherapy for Burkitt lymphoma with herpes labialis, and one patient with liver cirrhosis due to a chronic hepatitis C infection.

Identified confounders per alloimmunisation determinant are presented in Tables SII and SIII. As illustrated, control subjects with viral infections were younger, had received more red cell units, and were more often leucopenic as compared to those without viral infections. These differences were probably due to a higher frequency of haematological malignancies and associated treatment modalities.

Missing data for any identified confounder per determinant was at a maximum of 3·1%. CRP values were not measured in 343 patients (22·6%) during the risk period (Table SI).

The association between various types of infections and red cell alloimmunisation

The number of cases and controls diagnosed per type of infection are presented in Table III. For some patients, the

Table I. Patient characteristics during the alloimmunisation risk period.

Characteristics	Cases (N = 505)	Controls (N = 1010)	Missing data
Male	237 (46·9)	568 (56·2)	
Age, years (median, IQR)	67·0 (55·0–75·9)	65·3 (51·6–75·1)	
Transfused in a university hospital	232 (45·9)	464 (45·9)	
Cumulative (lifetime) number of red cell units up to implicated transfusion, median (IQR)	4 (2–8)	4 (2–8)	
Single transfused, N (%)	26 (5·1)	7 (0·7)	
Follow-up (days) up till last screen, median (IQR)	92 (20–193)	117 (10–609)	
Cumulative number of red cell units during risk period, median (IQR)	3 (2–6)	4 (2–8)	
Days transfused during risk period, median, (IQR)	1 (1–3)	2 (1–3)	
ICU admission	177 (36·5)	369 (35·0)	
Days at ICU, median (IQR)	7 (2–18)	7 (2–17)	4
Surgery	267 (52·9)	457 (45·2)	2
Thoracic including CABG	61 (12·1)	144 (14·3)	
Abdominal	100 (19·8)	181 (17·9)	
Back or spinal cord	3 (0·6)	11 (1·1)	
Diabetes mellitus type 1	6 (1·2)	7 (0·7)	
Diabetes mellitus type 2	91 (18·0)	176 (17·4)	1
Atherosclerosis*	198 (39·5)	314 (31·5)	17
Chronic obstructive airway disease†	43 (8·5)	89 (9·0)	20
Splenectomy (in past or during risk period)	1 (0·2)	19 (1·9)	
Organ transplant	4 (0·8)	23 (2·3)	
Liver cirrhosis	13 (2·6)	24 (2·4)	2
Haematological malignancy	60 (11·9)	210 (20·8)	13
Carcinoma	112 (22·3)	183 (18·2)	7
Chemotherapy	66 (13·1)	219 (21·8)	6
Radiotherapy	15 (3·0)	37 (3·6)	
Leucopenia‡	102 (20·2)	313 (31·0)	41
Haematopoietic stem cell transplantation (autologous or allogeneic, in past or during risk period)	10 (2·0)	63 (6·2)	
Graft versus host disease (acute or chronic)	4 (1·5)	15 (0·8)	3
Immunosuppressant medication§	154 (30·9)	423 (42·4)	20
GFR $\leq$ 30 ml/min¶	56 (11·1)	149 (14·8)	2
Dialysis (either chronic or acute)‖	31 (6·1)	98 (9·7)	

Values are n (%), unless otherwise stated. Numbers of patients for whom data on certain diagnoses and/or treatment modalities were not documented are presented as missing.

CABG, coronary artery bypass graft; GFR, glomerular filtration rate; ICU, intensive care unit; IQR, interquartile range.

*Systemic or coronary atherosclerosis.

†Chronic asthma bronchiale or chronic obstructive pulmonary disease.

‡At least one measured leucocyte count below lower limit of normal.

§Medication under subcategory H02 (corticosteroids) or L04 (other immunosuppressants) within the Anatomical Therapeutic Chemical (ATC) classification index (WHO Collaborating Centre for Drug Statistics Methodology, 2016).

¶GFR below 30 ml/min during at least 1 week of the risk period (with GFR calculated using the Modification of Diet in Renal Diseases [MDRD] equation; Levey *et al*, 1999).

‖Haemodialysis, peritoneal dialysis, or continuous veno-venous haemofiltration needed for at least 1 day during the risk period.

presence or absence of a certain type of infection could not be determined. The majority of these cases were due to an unestablished origin of the inflammatory condition (i.e. an infection or other inflammatory causes). In order to avoid misclassification, we omitted these patients from the corresponding analysis.

Mild bacterial infections were not associated with alloimmunisation. Patients with a severe tissue-invasive bacterial infection tended towards increased alloimmunisation risks [adjusted RR 1·34 (95% CI 0·97–1·85); Table III]. Relative risks increased to significance when these infections were accompanied with long-lasting fever [adjusted RR 3·06 (95% CI 1·57–5·96) with fever present for at least 7 days, Table IV]. The timing of fever i.e. occurring close to the implicated transfusion or at any time point during the risk period did not influence RRs (Table SIV). A sensitivity analysis on patients originally omitted from the analysis on severe bacterial infection (N = 47) did not change RRs (Table SV).

Given that alloantibodies against E, C^w, Le^a, Le^b, Lu^a, and M can also form 'naturally', [e.g. in response to microbial epitope exposure rather than to transfusion-related red cell exposure (Reid *et al*, 2004)] we evaluated a possible

Table II. Infections diagnosed during the alloimmunisation risk period.

(a) Locus of bacterial infections according to severity

Mild bacterial infections	*N*	Severe bacterial infections	*N*
Diagnosed in no. of patients	116	Diagnosed in no. of patients	269
Bacterial enteritis	12	Abdominal infections (including abscesses)	87
Catheter related*	37	Arthritis, bursitis, myositis, fasciitis, infected haematoma	11
Lower urinary tract infection	36	Bacterial meningitis	5
Skin and superficial wound infections	25	Deep wound or skin infection	20
Upper respiratory tract infection	11	Endocarditis, mediastinitis, pericarditis	21
		Infected foreign material	15
		Lower respiratory tract infection	85
		Non-abdominal abscesses	17
		Osteomyelitis, spondylodiscitis	5
		Upper urinary tract infection	19

(b) Microorganism genus (and species)

Gram-positive bacteraemia	*N*	Gram-negative bacteraemia	*N*
Diagnosed in no. of patients	117	Diagnosed in no. of patients	57
Bacillus	1	Bacteroides	4
Clostridium	1	Burkholdera	1
Corynebacterium	2	Capnocytophaga	1
Enterococcus	30	Enterobacter	8
Gemella	2	Escheria (Coli)	23
Listeria	1	Neisseria (Meningitides)	1
Micrococcus	1	Klebsiella	11
Staphylococcus	62	Proteus	2
Excluding coagulase negative	22	Pseudomonas	9
Streptococcus	25	Serratia	7
		Stenotrophomonas	1
Viral infections		**Fungal infections**	
Diagnosed in no. of patients	53	Diagnosed in no. of patients	56
Local viral infections		Aspergillus (pulmonary)	11
BK (cystitis)	1	Candida	42
HSV (stomatitis)	13	Stomatitis	10
Respiratory virus†	11	Candidaemia	11
Enteral virus‡	2	Other location	22
Disseminated viral diseases		Pneumocystis (jirovecii)	2
Adenoviraemia	3	Penicillium (pulmonary)	1
BK viraemia	1		
Cytomegalovirus viraemia	11		
Epstein Barr Virus viraemia	2		
Hepatitis C vireamia	6		
Human Herpesvirus- 6 viraemia	2		
Human immunodeficiency virus	3		
Varicella Zoster Virus reactivation	3		

Cumulative numbers per type of infection do not necessarily equal the number of patients diagnosed with this infection, as individual patients can have been infected with multiple microorganisms and types of infections.

*Routine (tip) cultures from central catheters and catheter induced phlebitis.

†Coronavirus (1), H1N1 virus (1), herpes simplex virus- 1 with bronchial location (1), influenza-virus (2), para-influenza virus (2), respiratory syncytial virus (1), rhinovirus (3).

‡Norovirus (1), rotavirus (1).

association between the induction of these antibodies and various infections using Pearson's chi-square test. The distribution of alloantibodies known to also occur 'naturally' did not differ between patients with and without severe bacterial infections (Table V).

Interestingly, patients with a Gram-negative bacteraemia tended to demonstrate reduced alloimmunisation rates [adjusted RR 0·58 (95% CI 0·13–1·14)], while Gram-positive bacteraemia was not associated with red cell alloimmunisation (Table III). To exclude a potential dilution of

Table III. Association between (various types of) bacterial and viral infections and red cell alloimmunisation.

Type of infection	Cases, *N*/total	Controls, *N*/total	RR (95% CI)*	Adjusted RR (95% CI)†	Excluded from analysis
Bacterial infections					
Tissue invasive infections	129/486	228/961	1·17 (0·90–1·51)	1·30 (0·98–1·74)	68
Mild	39/499	77/989	0·99 (0·66–1·49)	1·08 (0·70–1·66)	27
Severe	100/490	169/978	1·22 (0·92–1·62)	1·34 (0·97–1·85)	47
Bacteraemia	45/502	114/1003	0·75 (0·51–1·09)	0·89 (0·59–1·36)	10
Gram-positive	34/502	83/1003	0·78 (0·51–1·20)	1·08 (0·66–1·74)	10
Gram-positive, non-CNS	24/504	61/1009	0·82 (0·40–1·67)	0·96 (0·56–1·65)	2
Gram-negative	13/505	44/1010	0·57 (0·30–1·09)	0·58 (0·13–1·14)	0
Viral infections					
All	15/503	38/1003	0·72 (0·38–1·38)	1·56 (0·75–3·25)	9
Local	7/503	20/1003	0·71 (0·29–1·74)	1·80 (0·65–4·98)	9
Disseminated	10/505	20/1010	0·89 (0·40–2·02)	2·41 (0·89–6·53)	0
Fungal infections					
All	12/501	44/1001	0·50 (0·25–0·99)	0·60 (0·29–1·25)	13
Candidaemia	4/505	7/1010	1·19 (0·31–4·55)	2·93 (0·54–15·89)	0
Invasive aspergillus	1/503	10/1004	0·17 (0·02–1·42)	0·33 (0·03–3·28)	8

Patients for whom the presence or absence of a given infection could not be determined were excluded from the corresponding analysis.
RR, relative risk; 95% CI, 95% confidence interval; CNS, coagulase negative staphylococcus.
*Adjusted for: number of transfused red cell units and hospital.
†Additionally adjusted for identified potential confounders (for details, see Table SIII).

Table IV. Infections and red cell alloimmunisation according to the presence of fever and its duration.

Type of infection	Fever	Cases, *N*/total	Controls, *N*/total	RR (95% CI)*	Adjusted RR (95% CI)†
Severe bacterial infection					
–		390/490	809/978	ref.	ref.
+	–	17/490	48/978	0·72 (0·41–1·29)	0·79 (0·44–1·43)
+	1–6 days	59/490	101/978	1·20 (0·84–1·71)	1·33 (0·91–1·99)
+	≥7 days	24/490	20/978	2·67 (1·40–5·07)	3·06 (1·57–5·96)
Gram-positive bacteraemia					
–		468/502	921/1003	ref.	ref.
+	–	3/502	13/1003	0·51 (0·15–1·81)	0·88 (0·24–3·28)
+	1–6 days	21/502	54/1003	0·72 (0·42–1·22)	0·92 (0·52–1·61)
+	≥7 days	10/502	15/1003	1·29 (0·55–3·03)	2·14 (0·84–5·41)
Gram-negative bacteraemia					
–		492/505	966/1010	ref.	ref.
+	–	0/505	6/1010	0 (NC)	0 (NC)
+	1–6 days	12/505	34/1010	0·70 (0·35–1·39)	0·71 (0·35–1·45)
+	≥7 days	1/505	4/1010	0·52 (0·04–6·30)	0·53 (0·04–6·62)
Disseminated viral diseases					
–		495/505	990/1010	ref.	ref.
+	–	4/505	7/1010	1·14 (0·33–3·97)	1·89 (0·50–7·15)
+	1–6 days	4/505	9/1010	0·61 (0·16–2·38)	3·77 (0·64–22·24)
+	≥7 days	2/505	4/1010	1·12 (0·20–6·39)	2·58 (0·37–17. .82)

Only numbers of patients for whom the presence or absence of a given infection could be determined are presented.
RR, relative risk; 95% CI, 95% confidence interval; NC, not computable.
*Adjusted for: number of transfused red cell units and hospital.
†Additionally adjusted for identified potential confounders (for details, see Table SIII).

an existing effect by contaminated blood cultures positive for CNS, we also evaluated the association of non-CNS Gram-positive bacteraemia with alloimmunisation. RRs from this analysis were identical to the originally calculated RRs.

Any viral disease tended to be associated with increased red cell alloimmunisation incidences. The adjusted RR associated with disseminated viral infections was 2·41 (95% CI 0·89–6·53). The presence of fever did not influence RRs of viral infections (Table IV).

Table V. Specificity and frequency of first-formed red cell alloantibodies according to the presence of various types of infections.

Alloantibody specificity	All patients, *N* (%)	No infection, *N* (%)	Severe bacterial infection, *N* (%)	Viral infection (local and disseminated), *N* (%)	Gram-negative bacteraemia, *N* (%)
anti-C	23 (4·0)	19 (5·2)	1 (0·9)	0 (0)	1 (7·1)
anti-c	41 (7·2)	25 (6·8)	8 (7·1)	0 (0)	1 (7·1)
anti-E	185 (32·3)	113 (30·7)	41 (36·4)	4 (26·7)	5 (35·7)
anti-e	5 (0·9)	5 (1·4)	0 (0)	0 (0)	0 (0)
anti-K	126 (22·0)	88 (23·9)	21 (18·6)	3 (20·0)	6 (42·9)
anti-C^w	19 (3·3)	10 (2·7)	4 (3·5)	3 (20·0)	0 (0)
anti-Fy^a	31 (5·4)	24 (6·5)	4 (3·5)	1 (6·7)	0 (0)
anti-Fy^b	5 (0·9)	4 (1·1)	1 (0·9)	0 (0)	0 (0)
anti-Jk^a	54 (9·4)	37 (10·1)	8 (7·1)	3 (20·0)	0 (0)
anti-Jk^b	7 (1·2)	4 (1·1)	2 (1·8)	0 (0)	0 (0)
anti-Le^a	7 (1·2)	2 (0·5)	4 (3·5)	0 (0)	0 (0)
anti-Le^b	3 (0·5)	1 (0·3)	1 (0·9)	0 (0)	0 (0)
anti-Lu^a	32 (5·6)	19 (5·2)	9 (8·0)	0 (0)	0 (0)
anti-Lu^b	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
anti-M	22 (3·8)	14 (3·8)	5 (4·4)	1 (6·7)	0 (0)
anti-N	1 (0·2)	0 (0)	0 (0)	0 (0)	0 (0)
anti-S	12 (2·1)	7 (1·9)	4 (3·5)	0 (0)	1 (7·1)
anti-s	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
(possibly) natural occurring*	268 (46·7)	159 (43·2)	64 (56·6)	8 (53·3)	5 (35·7)
All antibodies	573	368	113	15	14
Number of patients	505	325	100	10	13

*Including: anti-E, anti-C^w, anti-Le^a, anti-Le^b, anti-Lu^a, and anti-M. No difference in distribution of (possibly) natural occurring alloantibodies was observed between patients with and without severe bacterial infections ($P = 0·08$), disseminated viral infections ($P = 0·93$), and Gram-negative bacteraemia ($P = 0·41$).

Fungal infections, as well as candidaemia and invasive aspergillus infections separately, were associated with heterogeneous RRs not reaching significance (Table III).

The association between laboratory indicators of inflammation and red cell alloimmunisation

Neither leucocytosis nor CRP level was associated with red cell alloimmunisation. A sensitivity analysis on parameters determined within the week following the implicated transfusion did not change these results (Table SVI).

Discussion

This study, the first of its kind, in transfused patients suggests a possible association between infectious conditions and red cell alloimmunisation. Specifically, our observations suggest alloimmunisation to be influenced by the type and intensity of, and the patient's inflammatory response to infections. In summary, severe (tissue-invasive) bacterial and viral infections were associated with increased incidences of alloimmunisation [RRs 1·34 (95% CI 0·97–1·85) and 2·41 (95% CI 0·89–6·53)]. In contrast, Gram-negative bacteraemia coincided with a twofold reduction of alloimmunisation risk [RR 0·58 (95% CI 0·13–1·14)].

Our findings certainly require additional confirmatory research. However, they seem to be biologically plausible and are in line with prior animal experiment observations (Hendrickson *et al*, 2006, 2007, 2008; Smith *et al*, 2012).

First, long-lasting fever with severe bacterial infections was associated with a substantially increased risk [RR 3·06 (95% CI 1·57–5·96)]. Here, persistence of fever could have reflected the most severe bacterial infections inducing a profound inflammatory response. Alternatively, or additionally, fever might have been due to other concomitant inflammatory conditions. Yet, both explanations are consistent with the 'danger model' (Matzinger, 1994) which postulates that an immune response is facilitated by pathogen-associated molecular patterns or structures released from cells undergoing stress (Matzinger, 1994; Gallucci & Matzinger, 2001; Kawai & Akira, 2010).

Second, although the 95% CI encompassing 1 (i.e. a null effect) warrants firm conclusions, we observed substantially increased alloimmunisation rates in patients with systemic viral infections. Murine experiments showed similar effects for poly(I:C) (Hendrickson *et al*, 2006, 2007, 2008; Smith *et al*, 2012), a synthetic viral RNA analogue that agonizes Toll-like receptor (TLR) 3 (Alexopoulou *et al*, 2001). These poly(I:C) effects were attributed to an increased dendritic cell consumption of transfused cells with upregulation of costimulatory molecules, and activation and proliferation of naive $CD4^+$ antigen-specific T-cells (Hendrickson *et al*, 2007, 2008). An existing molecular mimicry between certain viral peptides and $CD4^+$ T-cell red cell antigen epitopes was also

suggested, although observed effects in polyomavirus infected mice did not reach statistical significance (Hudson *et al*, 2010).

Although we did not analyse the association between latent viral infections and red cell alloimmunisation, these might also be relevant. In addition, the assessment of possible different effects of RNA and DNA viruses was prevented by low event numbers.

Third, we observed a twofold alloimmunisation incidence reduction during Gram-negative bacteraemia. Analogous to viral infections, these findings require confirmatory research. Yet, they again corroborate animal experiments showing significantly attenuated alloimmunisation responses upon lipopolysaccharide (LPS) pretreatment in mice (Hendrickson *et al*, 2008). LPS, an endotoxin in the outer cell membrane of Gram-negative bacteria, strongly stimulates innate immunity by agonizing TLR4 on macrophages and dendritic cells. Conversely, LPS is also implicated in a transient, possibly self-protective immune paralysis, known as LPS tolerance (Lauw *et al*, 2000; Weijer *et al*, 2002; Gould *et al*, 2004). Restimulation with LPS in this respect initiates blockage of $CD4^+$ T cell functioning via impaired release of tumour necrosis factor α, interleukin (IL)12 and IL18 from monocytes and dendritic cells, together with a diminished upregulation of major histocompatibility complex class-II and costimulatory molecules (Mattern *et al*, 1998; Gould *et al*, 2004). While regulatory T cells selectively express TLRs (including TLR4), their LPS-induced proliferation might also contribute to the observed effects in both mice and humans (Caramalho *et al*, 2003). Finally, we cannot exclude an indirect role for Gram-negative bacteraemia on red cell alloimmunisation due to their common association with other modulators. Indeed, suppressed mitogenic B and T lymphocyte responses were observed following administration of antibiotics, including cephalosporins, an antibiotic class frequently used in the treatment of Gram-negative bacterial infections (Borowski *et al*, 1985; Pomorska-Mol *et al*, 2015).

In an intriguing contrast to the effects observed for Gram-negative bacteraemia, we did not observe any association between Gram-positive bacteraemia and red cell alloimmunisation. A common lower degree of acute inflammation evoked by gram-positive as compared to gram-negative bloodstream infections due to differing virulence mechanisms forms one hypothetical explanation (Wang *et al*, 2003; Gould *et al*, 2004; Abe *et al*, 2010).

Despite the RRs for fungal infections not differing significantly from those for Gram-negative bacteraemia, the heterogeneous RRs for individual fungal microorganisms and the lack of other supportive evidence prevent tentative inferences. Indeed, in contrast to our estimated RR, one report suggested neonatal alloimmunisation to be related to a disseminated histoplasmosis infection (Hata *et al*, 2013).

The ultimate goal of our study would be to establish an accurate alloimmunisation prediction model, serving as a practical tool for risk-based extended matching. Such a model would be most feasible when based on routinely measured patient parameters. In this perspective, we did not observe any association of the level of leucocytosis and CRP with alloimmunisation, possibly due to the multifactorial nature of these parameters. Other biomarkers, e.g. cytokine levels and immune cell subsets, might be better discriminative; however, they could not be evaluated in the current study.

Our study design, results and interpretations require additional remarks:

First, our incidence-density sampling strategy guarantees that selected controls were similarly exposed as their matched cases (Rothman, 2007). Hereby, our RRs are not influenced by transfusion burden, being a main determinant of red cell alloimmunisation (Zalpuri *et al*, 2012b; Evers *et al*, 2016).

Second, by identifying the implicated transfusion, we could study conditions present at that given time. As the duration of alloimmunisation modulation is currently unknown and will also probably differ per risk factor, we chose a seemingly large risk period to precede the implicated transfusion. Although one could argue that this strategy could possibly dilute some effects, on the other hand, it assures inclusion of most factors of influence at the time of exposure. For example, repeated LPS exposure might induce a state of tolerance persisting for up to 30 days (Cross, 2002). In addition, a recent study showed that poly(I:C) facilitates red cell alloimmunisation for at least 14 days with its maximum effect reached 7 days after administration (Elayeb *et al*, 2016). As a validation of our chosen risk period length, a sensitivity analysis on infections diagnosed during the week preceding or following the implicated transfusion did not change our conclusions (data not shown). Similarly, only the duration of fever accompanying severe bacterial infections, rather than its timing in the risk period, affected alloimmunisation. As we aimed to target the most likely first initiation of an alloimmune response, we limited the risk period to the first 7 days following the implicated transfusion.

Third, actual lag periods per antigen-specific antibody are currently unknown. As such, our chosen lag period of 7 days might not completely have prevented the exclusion of patients demonstrating recall responses, including women immunised due to prior pregnancies. Direct antiglobulin tests were not performed on a routine basis shortly following transfusion and as such were of no help in identifying these patients. However, as non-RhD alloantibodies form in only 0·33% of first trimester pregnancies (Koelewijn *et al*, 2008), we believe that a substantial influence of previous pregnancies is unlikely. Moreover, erroneously considering a substantial amount of boosting reactions as primary alloimmunisation events would have biased our RRs towards the null-effect. Indeed, a sensitivity analysis in which we excluded the 53 patients in whom alloantibodies were discovered during the second week following their first antigen-incompatible transfusion did not substantially change RRs (data not shown). In conclusion, we believe the eventual bias due to our choice of the lag period to be small.

Fourth, to avoid invalid inferences due to misclassification, we did not define patients with a non-established aetiology of their inflammatory phenotype as exposed patients. For example, for a vascular compromised patient diagnosed with osteomyelitis, wound cultures positive for *Staphylococcus Aureus* might have represented normal skin flora colonization of a primary ischaemic wound. Consequently, the analysis on severe bacterial infections did not include this patient. A sensitivity analysis confirmed our results not to be affected by this possible misclassification bias.

In conclusion, our data suggest a potential risk modifying influence of infection-associated inflammation on red cell alloimmunisation in transfused patients. Alloimmunisation seems to be induced by severe bacterial or viral infections, but might be skewed towards protection in the presence of Gram-negative bacteraemia. Further confirmatory research is needed to ultimately identify the high-risk patient and, consequently, better target the allocation of more extensively matched red cell units.

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Authorship

JJZ and JGB designed the study. DE, JT, and SZ collected the data. DE, JJZ, RAM, MH and JGB analysed and interpreted the data. DE, JJZ, MH, and JGB wrote the manuscript. All other authors revised and approved the final manuscript.

Disclosure of conflicts of interest

The authors declare that they have no conflict of interest relevant to the work presented in this manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Study period per participating hospital and the used multiple imputation model.

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