Clinical determinants of red cell alloimmunizatiom, implications for preventative antigen matching strategies

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RED CELL ALLOIMMUNIZATION IN PATIENTS WITH DIFFERENT TYPES OF INFECTIONS

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Abstract

Red cell alloantigen exposure can cause alloantibody associated morbidity. Murine models have suggested inflammation to modulate red cell alloimmunization. This study quantifies alloimmunization risks during infectious episodes in humans.

We performed a multicenter case-control study within a source population of patients receiving their first and subsequent red cell transfusions during an eight year follow-up period. Patients developing a first transfusion-induced red cell alloantibody (N=505) were each compared with two similarly exposed, but non-alloimmunized controls (N=1,010) during a five week 'alloimmunization 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 (CI) 0.97-1.85), especially when these infections were accompanied with long-standing fever (RR 3.06, CI 1.57-5.96). Disseminated viral disorders demonstrated a trend towards increased risks (RR 2.41, CI 0.89-6.53), in apparent contrast to a possible protection associated with Gram-negative bacteremia (RR 0.58, CI 0.13-1.14). 'Simple' bacterial infections, Gram-positive bacteremia, fungal infections, maximum CRP values, and leukocytosis were not associated with red cell alloimmunization.

These findings are consistent with murine models. Confirmational research is needed before patients likely to develop alloantibodies may be identified based on their infectious conditions at time of transfusion.

Introduction

Red cell alloimmunization challenges providing compatible donor blood and, most importantly, might induce severe hemolytic transfusion reactions.[1, 2] Consequently, some selected patients receive extended matched blood.[2, 3] Despite the effectiveness of these risk-based matching practices,[4-6] non-selected patients do experience alloimmunization-mediated complications[1, 2, 7] warranting consideration of additional risk factors.

Next to the chance to encounter a high immunogenic non-self antigen,[8] clinical conditions affecting the recipient's immune response likely modulate alloimmunization. Identification of such factors might enable allocating extended matched blood principally to high risk patients.

Experimentally induced inflammation has consistently been marked as a major determinant of red cell alloimmunization in mice.[9-12] In line, pro-inflammatory conditions related to sickle cell disease as well as febrile reactions to donor platelets were shown to enhance alloimmunization in humans.[13, 14] Apart from one case report,[15] to the best of our knowledge, the influence of infection-associated inflammation on red cell alloimmunization in humans has not been reported.

In this nested case-control study, we quantify relative alloimmunization 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.

Methods

Study design and setting

We performed a nested case-control study within a source population of previously non-transfused and non-alloimmunized patients in three university and three reference hospitals in the Netherlands. Using this design, we compared patients who developed red cell alloantibodies following transfusion with non-alloimmunized 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.[8, 16]

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 necessary data between January 1, 2005 and December 31, 2013 (for details, see supplementary box 1). All red cell units were prepared by buffy-coat depletion of whole blood donations, subsequently filtered through a leukocyte depletion filter, and stored in SAGM for a maximum of 35 days.[3]

Patients were defined as 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, Le^a, Le^b, Lu^a, Lu^b, M, N, S, or s. Anti-D immunized patients were not taken into consideration since 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-immunized patients) were deemed ineligible. In addition, alloimmunized patients were excluded if their first-time alloantibody positive screen occurred within seven days of the first mismatched transfusion, as these more likely represented boosting to earlier primary immunizations. By consulting the nationwide alloimmunization registry,[17] we additionally excluded patients previously diagnosed with alloimmunization in other hospitals. Considering the above mentioned criteria, we specifically aimed to exclude previously alloimmunized patients, including pregnancy-induced immunizations in women. Finally, hemoglobinopathy patients and infants below six months of age were not included.

Each eligible case was matched to two randomly selected non-alloimmunized control patients based on the hospital and on the (lifetime) number of red cell transfusions received at the time of alloimmunization. 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.[18]

For all cases, we assumed that the last antigen-mismatched transfusion (the '*Nth*' or implicated transfusion) preceding the first positive screen most likely elicited alloimmunization. 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 seven days to have elicited alloimmunization. An 'alloimmunization risk period' was then constructed stretching from 30 days before up to seven 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 alloimmunization risk period are illustrated in Figure 1.

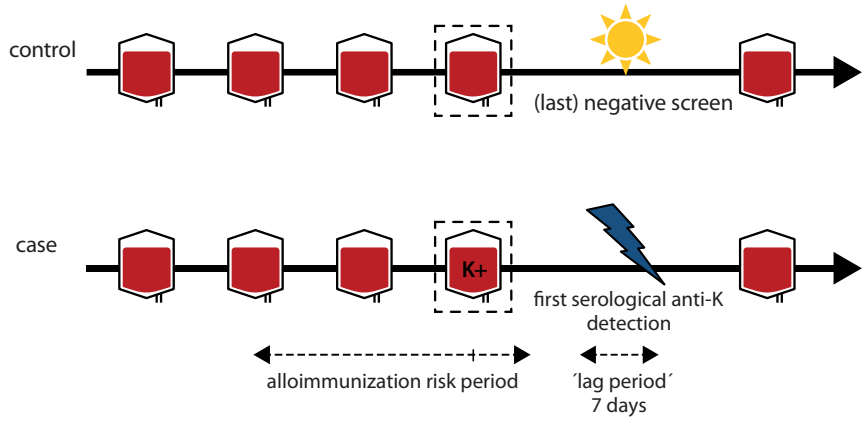
For all cases and controls, we recorded various clinical conditions during the alloimmunization risk period.

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

First-formed red cell alloantibodies

At a maximum of 72 hours prior to red cell transfusion, patients in the Netherlands are routinely screened for red cell alloantibodies. According to the Dutch transfusion guideline, 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 K antigen needs to be present

Figure 1 The implicated transfusion and alloimmunization 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 most likely influenced alloimmunization. To exclude possible boosting events, this implicated transfusion was required to precede the first positive screen by at least seven days (i.e. lag period). An alloimmunization 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 alloimmunization risk period around the *Nth* matched transfusion was constructed.

minimally heterozygously. The presence of C^w, Lu^a, Wr^a, and Kp^a is not mandatory on commercially available screening cells.[3] Antibody screening involves a three-cell panel using an indirect antiglobulin test (column agglutination technology from BioRad, Cressier, Switzerland, or from Ortho Clinical Diagnostics, Raritan NJ, United States). If positive, screening is followed by subsequent 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 leukocyte 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 alloimmunization risk period, including dates of infection, the causative microorganisms, dates of fever (temperature ≥38.5 °C), leukocyte counts, and CRP values.

Bacterial infections comprised tissue-invasive infections (i.e. involving an anatomic site location) and bacteremia (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 hematoma, bacterial meningitis, deep wound or skin infections, endocarditis, mediastinitis, pericarditis, infected foreign material, lower respiratory tract infections, osteomyelitis, spondylodiscitis, and upper urinary tract infections.

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

For the qualification of a viral infection, a positive 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. Viremia and disseminated viral zoster infections were defined as 'disseminated viral infections', contrasting 'local viral infections' restricted to one anatomic site location.

Statistical analyses

The associations of various infections with the development of red cell alloimmunization 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. alloimmunization), and is not in the causal pathway between the exposure.[19, 20] 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-alloimmunized 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.[19] 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.[21] Third, to minimize bias due to missing data on the confounders, we used multiple imputation. Details on the used model can be found in the Supplementary Box 2. Finally, we evaluated the association between various types of infections and red cell alloimmunization by subsequently entering the corresponding probability scores into the logistic regression model with alloimmunization as the outcome and conditioning on the matched variables.

We next assessed the association of level of CRP values and leukocytosis as possible markers of inflammation with red cell alloimmunization. Leukocytosis was categorized as maximum measured leukocyte counts of 10-15, 15-20, 20-30, and >30x10^9/L, and referenced to normal counts (4-10x10^9/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 leukocyte value 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 leukocytosis reflect various clinical conditions preventing causal inferences, we present here only 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),[22] 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,[18] we interpreted and present all odds ratios as RR with 95% confidence intervals (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 hospitalized patients, with *Streptococcus Pneumoniae* and *Haemophilus Influenzae* alone representing 30-75% of causative pathogens.[23]

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

Results

Among 54,347 newly-transfused patients, 24,063 were considered eligible (Figure S1) of which 505 patients (2.1%) formed red-cell alloantibodies. Thirty-seven of these alloimmunized 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 alloimmunization.

General and clinical characteristics of the 505 cases and their 1,010 matched controls during the alloimmunization risk period are presented in Table 1.

Infections during the alloimmunization risk period

Among all cases and controls, 473 patients were diagnosed with at least one infection during the alloimmunization risk period. Of these, 417 suffered from bacterial infections, 53 from viral infections, and 56 from fungal infections (Table 2).

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 alloimmunization 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 a Burkitt lymphoma with herpes labialis, and one patient with liver cirrhosis due to a chronic hepatitis C infection.

Identified confounders per alloimmunization determinant are presented in Table S1 and S2. As illustrated, control subjects with viral infections were younger, had received more red cell units, and were more often leukopenic as compared to those without viral infections. These differences were likely due to a higher frequency of hematological malignancies and associated treatment modalities.

Missing data for any identified confounder per determinant was maximally 3.1%. For 343 patients (22.6%), CRP values were not measured during the risk period (Table S3).

The association between various types of infections and red cell alloimmunization

Table 3 presents the number of cases and controls diagnosed per type of infection. For some patients, the 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. being due to infection or other inflammatory causes). In order to avoid misclassification, we omitted these patients from the corresponding analysis.

Mild bacterial infections were not associated to alloimmunization. Patients with a severe tissue-invasive bacterial infection tended towards increased alloimmunization risks (adjusted RR 1.34 (CI 0.97-1.85), Table 3). Relative risks increased to significance when these infections were accompanied with long-lasting fever (adjusted RR 3.06 (CI 1.57-5.96) with

Table 1 Patient characteristics during the alloimmunization risk period.

Characteristics	Cases (N=505)	Controls (N=1,010)	Missing
Men	237 (46.9)	568 (56.2)	
Age in years (median, IQR)	67.0 (55.0-75.9)	65.3 (51.6-75.1)	
Transfused in university hospitals	232 (45.9)	464 (45.9)	
Cumulative (lifetime) number of red cell units up till implicated transfusion (median, IQR)	4 (2-8)	4 (2-8)	
Single transfused (N, %) follow-up (days) up till last screen (median, IQR)	26 (5.1) 92 (20-193)	7 (0.7) 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 days at ICU (median, IQR)	177 (36.5) 7 (2-18)	369 (35.0) 7 (2-17)	 4
Surgery thoracic including CABG abdominal back or spinal cord	267 (52.9) 61 (12.1) 100 (19.8) 3 (0.6)	457 (45.2) 144 (14.3) 181 (17.9) 11 (1.1)	2
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
Hematological 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)	
Leukopenia ‡	102 (20.2)	313 (31.0)	41
Hematopoietic 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 ≤ 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.

IQR = interquartile range. * systemic or coronary atherosclerosis. † chronic asthma bronchiale or chronic obstructive pulmonary disease. ‡ at least once measured leukocyte counts below lower limit of normal. § medication under subcategory H02 (corticosteroids) or L04 (other immunosuppressants) within the Anatomical Therapeutic Chemical (ATC) classification index. ‖ glomerular filtration rate (GFR) below 30 ml/min during at least one week of the risk period (with GFR calculated using the Modification of Diet in Renal Diseases (MDRD) equation). ¶ hemodialysis, peritoneal dialysis, or continuous veno-venous hemofiltration needed for at least one day during the risk period.

Table 2 Infections diagnosed during the alloimmunization risk period.

A Locus of bacterial infections according to severity

Mild bacterial infections	N	Severe bacterial infections	N
Diagnosed in *N* patients	116	Diagnosed in *N* patients	269
Bacterial enteritis	12	Abdominal infections (including abscesses)	87
Catheter related *	37		
Lower urinary tract infection	36	Arthritis, bursitis, myositis, fasciitis, infected hematoma	11
Skin and superficial wound infections	25	Bacterial meningitis	5
Upper respiratory tract infection	11	Deep wound or skin infection	20
		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 bacteremia	N	Gram-negative bacteremia	N
Diagnosed in *N* patients	117	Diagnosed in *N* 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 *N* patients	53	Diagnosed in *N* patients	56
Local viral infections		Aspergillus (pulmonary)	11
BK (cystitis)	1	Candida	42
HSV (stomatitis)	13	stomatitis	10
Respiratory virus †	11	candidemia	11
Enteral virus ‡	2	other location	22

Table 2 Continued.

B Microorganism genus (and species)

Viral infections		Fungal infections	
Disseminated viral diseases		Pneumocystis (jirovecii)	2
Adenoviremia	3	Penicillium (pulmonary)	1
BK viremia	1		
Cytomegalovirus viremia	11		
Epstein Barr Virus viremia	2		
Hepatitis C viremia	6		
Human Herpesvirus- 6 viremia	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).

fever present for at least seven days, Table 4). 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 (data not shown). RRs from a sensitivity analysis in which patients originally omitted from the analysis on severe bacterial infection (N=47) were alternately assigned to exposure and non-exposure of this infection did not differ (RR 1.26 (CI 0.93-1.71) versus 1.33 (0.97-1.83), respectively).

Since 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),[22] we evaluated a possible 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 5).

Interestingly, patients with a Gram-negative bacteremia tended to demonstrate reduced alloimmunization rates (adjusted RR 0.58, (CI 0.13-1.14)), while Gram-positive bacteremia was not associated with red cell alloimmunization (Table 3). To exclude a potential dilution of an existing effect by contaminated blood cultures positive for CNS, we in addition evaluated the association of non-CNS Gram-positive bacteremia with allo-immunization. RRs from this analysis were identical to originally calculated RRs.

Any viral disease tended to be associated with increased red cell alloimmunization incidences. The adjusted RR associated with disseminated viral infections was 2.41 (CI 0.89-6.53). The presence of fever did not influence RRs of viral infections (Table 4).

Table 3 Association between (various types of) bacterial and viral infections and red cell alloimmunization.

Type of infection	Cases, N/total	Controls, N/total	RR (CI) *	Adjusted RR (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
bacteremia	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
candidemia	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.

* Adjusted for: number of transfused red cell units and hospital. † Additionally adjusted for identified potential confounders (for details, see Table S2). RR = relative risk. CI = 95% confidence interval. CNS = coagulase negative staphylococcus.

Fungal infections, as well as candidemia and invasive aspergillus infections separately, were associated with heterogeneous RRs not reaching significance (Table 3).

The association between laboratory indicators of inflammation and red cell alloimmunization

Neither leukocytosis nor CRP value was associated with red cell alloimmunization (Table S4). A sensitivity analysis on parameters determined within the week following the implicated transfusion did not change results (Table S4).

Table 4 Infections and red cell alloimmunization according to the presence of fever and its duration.

Type of infection	Fever	Cases, N/total	Controls, N/total	RR (CI) *	Adjusted RR (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 bacteremia					
-		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 bacteremia					
-		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. * Adjusted for: number of transfused red cell units and hospital. † Additionally adjusted for identified potential confounders (for details, see Table S2). RR = relative risk. CI = 95% confidence interval. NC = not computable.

Table 5 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 bacteremia, 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 bacteremia (p=0.41).

Discussion

This first study of its kind in transfused patients suggests a possible association between infectious conditions and red cell alloimmunization. Specifically, our observations suggest alloimmunization 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 alloimmunization incidences (RRs 1.34 (CI 0.97-1.85) and 2.41 (CI 0.89-6.53)). In contrast, Gram-negative bacteremia coincided with a 2-fold reduction of alloimmunization risk (RR 0.58 (CI 0.13-1.14)).

Our findings certainly require additional confirmational research. However, they seem biological plausible and are in line with prior animal experiment observations.

First, long-lasting fever with severe bacterial infections was associated with a substantially increased risk (RR 3.06 (CI 1.57-5.96)). Here, persistence of fever could have reflected the most severe bacterial infections inducing a profound inflammatory response. Alternately or additionally, fever might have been due to other concomitant inflammatory conditions. Yet, both explanations are consistent with the 'danger model' which postulates that an immune response is facilitated by pathogen associated molecular patterns or structures released from cells undergoing stress.[24-26]

Second, although the 95% confidence interval encompassing 1 (i.e. a null effect) warrants firm conclusions, we observed substantially increased alloimmunization rates in patients with systemic viral infections. Murine experiments showed similar effects for poly(I:C),[9-12] a synthetic viral RNA analogue which agonizes Toll-like receptor (TLR)-3.[27] 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.[9, 10] An existing molecular mimicry between certain viral peptides and $CD4^+$ T cell red cell antigen epitopes was also suggested, albeit observed effects in polyomavirus infected mice did not reach statistical significance.[28]

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

Third, we observed a 2-fold alloimmunization incidence reduction during Gram-negative bacteremia. Analogous to viral infections, these findings require confirmational research. Yet, they again corroborate animal experiments showing significantly attenuated alloimmunization responses upon lipopolysaccharide (LPS) pretreatment in mice.[10] LPS, an endotoxin in the outer cell membrane of Gram-negative bacteria, strongly stimulates innate immunity by agonizing TLR-4 on macrophages and dendritic cells. Conversely, LPS is also implicated in a transient, possibly self-protective immune paralysis, known as LPS tolerance.[29-31] Restimulation with LPS in this respect initiates blockage of $CD4^+$ T cell functioning via impaired release of TNFα, IL-12, and IL-18 from monocytes and dendritic cells together with a diminished upregulation of MHC class-II and costimulatory

molecules.[29, 32] While regulatory T cells selectively express TLRs (including TLR-4), their LPS induced proliferation might also contribute to the observed effects in both mice and human.[33] Finally, we cannot exclude an indirect role for Gram-negative bacteremia on red cell alloimmunization 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.[34, 35]

In intriguing contrast to the effects observed for Gram-negative bacteremia, we did not observe any association between Gram-positive bacteremia and red cell alloimmunization. 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.[29, 36, 37]

Despite RRs for fungal infections not significantly differing from those for Gram-negative bacteremia, the heterogeneous RRs for individual fungal microorganisms and the lack of other supportive evidence prevent tentative inferences. Indeed, contrasting our estimated RR, one report suggested neonatal alloimmunization to be related to a disseminated histoplasmosis infection.[15]

The ultimate goal of our study would be to establish an accurate alloimmunization 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 leukocytosis and CRP values with alloimmunization, possibly due to the multifactorial nature of these parameters. Other biomarkers e.g. cytokine levels and immune cell subsets might be better discriminative, yet, 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.[18] Hereby, our RRs are not influenced by transfusion burden, being a main determinant of red cell alloimmunization.[8]

Second, by identifying the implicated transfusion, we could study conditions present at that given time. Since the duration of alloimmunization modulation is currently unknown and will also likely differ per risk factor, we chose a seemingly large risk period to precede the implicated transfusion. Although one could argue this strategy to possibly dilute some effects, it on the other hand 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.[38] In addition, a recent study showed that poly(I:C) facilitates red cell alloimmunization for at least 14 days with its maximum effect reached seven days after administration.[39] 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 alloimmunization. As we aimed to target the most likely first initiation of an alloimmune response, we limited the risk period to the first seven days following the implicated transfusion.

Third, actual lag periods per antigen-specific antibody are currently unknown. As such, our chosen lag period of seven days might not completely have prevented the exclusion of patients demonstrating recall responses, including women immunized due to prior pregnancies. Direct antiglobulin tests were not performed on a routine base 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,[40] we believe substantial influence of previous pregnancies unlikely. Moreover, erroneously considering a substantial amount of boosting reactions as primary alloimmunization 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 etiology 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 ischemic 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 alloimmunization in transfused patients. Alloimmunization seems induced with severe bacterial or viral infections, but might be skewed towards protection in the presence of Gram-negative bacteremia. Further confirmational research is needed to ultimately identify the high-risk patient and, consequently, better target the allocation of more extended matched red cell units.

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Supplementary material

Supplementary Box 1

The study period varied per hospital according to the electronic availability of necessary data: January 1, 2005 to December 31, 2010 at Leiden University Medical Center (Leiden), September 6, 2006 to December 31, 2013 at University Medical Center Utrecht (Utrecht), November 19, 2011 to December 31, 2013 at VU University Medical Center (Amsterdam), May 1, 2007 to April 30, 2013 at Catharina Hospital (Eindhoven), July 1, 2005 to December 31, 2013 at Jeroen Bosch Hospital ('s Hertogenbosch), and October 5, 2008 to December 31, 2013 at Haga Teaching Hospital (The Hague).

Supplementary Box 2

To provide values for some missing predictor values, we performed multiple imputation creating five imputed datasets. Predictor variables included: alloimmunization status, age, sex, number of transfusions received, (types of) infection, (duration of) fever, (duration of) admittance at the intensive care unit, (types of) surgery, (types of) malignancies, chemotherapy treatment, radiotherapy treatment, use of immunosuppressant medication, (timing of) allogeneic and/or autologous stem cell transplantation, graft versus host disease, diabetes mellitus type 1, diabetes mellitus type 2, atherosclerosis, liver cirrhosis, renal insufficiency with a GFR ≤ 30 ml/min, measured minimum leukocyte counts, measured maximum leukocyte counts, and measured maximum CRP values.

Figure S1 Flow diagram of source population establishment.

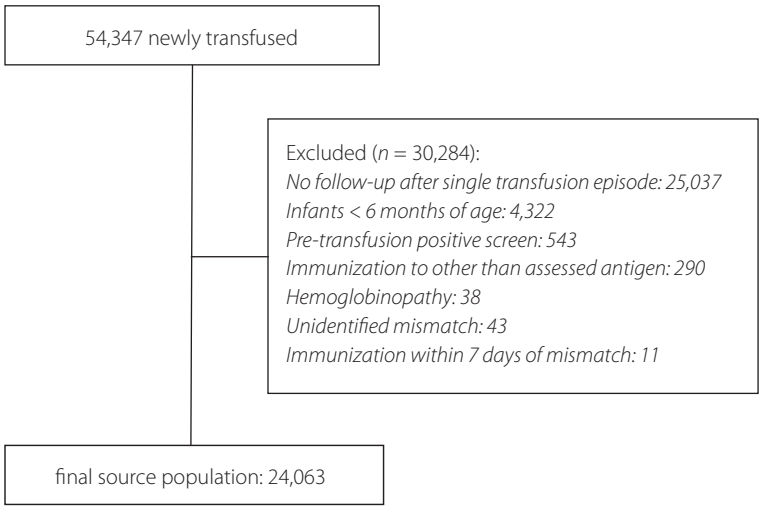

Figure adapted from: Evers, D., Middelburg, R.A., de Haas, M., Zalpuri, S., de Vooght, K. M., Visser, O., Péquériaux, N.C., Hudig, F., Schonewille, H., Zwaginga, J.J. Red cell alloimmunization in relation to antigens' exposure and their immunogenicity: a cohort study. *Lancet Haematol.* 2016;3(6):e284-92.

Table S1 Characteristics of 1,010 non-alloimmunized sampled controls during the alloimmunization risk period according to exposure of various infections.

Characteristics	Gram-negative bacteremia		Severe bacterial infection		Disseminated viral infection	
	present (N=44)	not present (N=966)	present (N=169)	not present (N=809)	present (N=20)	not present (N=990)
Men	27 (61.4)	540 (55.9)	110 (65.1)	443 (54.8)	10 (50.0)	558 (56.3)
Age in years (median, IQR)	65.0 (48.8-72.6)	65.3 (51.5-75.2)	66.7 (54.7-75.1)	64.6 (50.4-75.1)	44.1 (17.0-57.0)	65.4 (52.0-75.4)
Transfused in university hospitals	26 (59.1)	438 (45.3)	79 (46.7)	368 (45.5)	20 (100.0)	444 (44.8)
Cumulative (lifetime) number of red cell units received up till implicated transfusion (median, IQR)	6 (4-12)	4 (2-8)	5 (2-10)	4 (2-8)	7 (4-11)	4 (2-8)
Cumulative number of red cell units during risk period (median, IQR)	8 (4-14)	4 (2-8)	6 (4-11)	4 (2-8)	6 (4-11)	4 (2-8)
Days transfused during risk period (median, IQR)	4 (2-5)	2 (1-3)	3 (2-4)	2 (1-3)	4 (2-6)	2 (1-3)
ICU admission	30 (68.2)	339 (35.1)	113 (66.9)	230 (28.4)	7 (35.0)	362 (36.6)
days at ICU (median, IQR)	20.5 (6.8-35.0)	7.0 (2.0-14.0)	11 (6.0-28.5)	5.5 (2.0-9.0)	7.0 (1.0-27.5)	7.0 (2.0-17.0)
Surgery						
thoracic, including CABG	7 (15.9)	137 (14.2)	36 (21.3)	94 (11.6)	0 (0.0)	144 (14.5)
abdominal	16 (36.4)	165 (17.1)	61 (36.1)	113 (14.0)	2 (10.0)	179 (18.1)
back or spinal cord	0 (0)	11 (1.1)	3 (1.8)	8 (1.0)	0 (0)	11 (1.1)
Diabetes mellitus type 1	0 (0)	7 (0.7)	2 (1.2)	4 (0.5)	0 (0)	7 (0.7)
Diabetes mellitus type 2	9 (20.5)	167 (17.3)	32 (18.9)	135 (16.7)	1 (5.0)	175 (17.7)
Atherosclerosis *	11 (25.0)	303 (31.4)	57 (33.7)	243 (30.0)	1 (5.0)	313 (31.6)
Chronic obstructive airway disease †	5 (11.4)	84 (8.7)	25 (14.8)	59 (7.3)	0 (0)	89 (9.0)
Splenectomy (in past or during risk period)	1 (2.3)	18 (1.9)	8 (4.7)	10 (1.2)	1 (5.0)	18 (1.8)
Organ transplant	1 (2.3)	22 (2.3)	1 (0.6)	17 (2.1)	0 (0)	23 (2.3)
Liver cirrhosis	1 (2.3)	23 (2.4)	5 (3.0)	17 (2.1)	1 (5.0)	23 (2.3)
Hematological malignancy	8 (18.2)	202 (20.9)	7 (4.1)	198 (24.5)	5 (25.0)	205 (20.7)
Carcinoma	9 (20.5)	174 (18.0)	33 (19.5)	145 (17.9)	0 (0)	183 (18.5)

Chemotherapy	8 (18.2)	211 (21.8)	9 (5.3)	204 (25.2)	5 (25.0)	214 (21.6)
Radiotherapy	1 (2.3)	38 (3.9)	5 (3.0)	34 (4.2)	1 (5.0)	38 (3.8)
Leukopenia ‡	14 (31.8)	294 (30.4)	32 (18.9)	266 (32.9)	14 (70.0)	294 (29.7)
Hematopoietic cell transplantation (autologous or allogeneic, in past or during risk period)	1 (2.3)	63 (6.5)	4 (2.4)	60 (7.4)	14 (70.0)	50 (5.1)
Graft versus host disease (acute or chronic)	1 (2.3)	14 (1.4)	1 (0.6)	14 (1.7)	5 (25.0)	10 (1. 0)
Immunosuppressant medication §	27 (61.4)	396 (41.0)	89 (52.7)	311 (38.4)	18 (90.0)	405 (40.9)
GFR ≤ 30 ml/min ‖	8 (18.2)	141 (14.6)	48 (28.4)	92 (11.4)	4 (20.0)	145 (14.6)
Dialysis (either chronic or acute) ¶	7 (15.9)	91 (9.4)	38 (22.5)	55 (6.8)	2 (10.0)	96 (9.7)
Infections						
mild bacterial	7 (15.9)	70 (7.2)	18 (10.7)	55 (6.8)	3 (15.0)	74 (7.5)
severe bacterial	27 (61.4)	142 (14.7)	n.a.	n.a.	3 (15.0)	166 (16.8)
Gram-positive bacteremia	13 (28.5)	70 (7.2)	37 (21.9)	44 (5.4)	5 (25.0)	78 (7.9)
Gram-negative bacteremia	n.a.	n.a.	27 (16.0)	15 (1.9)	0 (0)	44 (4.4)
viral disseminated	0 (0)	20 (2.1)	3 (1.8)	17 (2.1)	n.a.	n.a.
fungal	6 (13.6)	38 (3.9)	20 (11.8)	22 (2.8)	4 (20.0)	40 (4.0)

Values are n (%), unless otherwise stated. IQR = interquartile range. n.a.= not applicable.
The presence or absence of a severe bacterial infection could not be determined for 32 control subjects.
* systemic or coronary atherosclerosis. † chronic asthma bronchiale or chronic obstructive pulmonary disease. ‡ at least once leukocyte count measured below lower limit of normal value. § medication under subcategory H02 (corticosteroids) or L04 (other immunosuppressants) within the Anatomical Therapeutic Chemical (ATC) classification index. ‖ glomerular filtration rate (GFR) below 30 ml/min during at least during one week of the risk period (calculated using the Modification of Diet in Renal Diseases (MDRD) equation). ¶ hemodialysis, peritoneal dialysis, or continuous venovenous hemofiltration needed for at least one day during the risk period.

Table S2 Subset of variables defined as confounders per determinant for alloimmunization.

Determinant	Confounders
all below determinants	age, gender, (duration of) ICU admittance, (type of) hematologic malignancy, chemotherapy, (degree of) leukopenia, immunosuppressant medication, GFR ≤ 30 ml/min.
mild or severe (tissue invasive) bacterial infection	thoracic surgery, abdominal surgery, diabetes mellitus type 2, COPD, carcinoma, (timing of) HSCT, dialysis, Gram-positive bacteremia, Gram-negative bacteremia, fungal infection.
mild bacterial infection	thoracic surgery, diabetes mellitus type 2, organ transplant, carcinoma, dialysis, Gram-positive bacteremia, Gram-negative bacteremia, mild bacterial infection, disseminated viral infection, fungal infection.
severe bacterial infection	thoracic surgery, abdominal surgery, atherosclerosis, COPD, splenectomy in past or during risk period, (timing of) HSCT, dialysis, Gram-positive bacteremia, Gram-negative bacteremia, mild bacterial infection, fungal infection.
bacteremia (all types)	thoracic surgery, abdominal surgery, diabetes mellitus type 2, carcinoma, dialysis, mild bacterial infection, severe bacterial infection, local viral infection, disseminated viral infection, fungal infection.
Gram-positive bacteremia	thoracic surgery, diabetes mellitus type 2, carcinoma, (timing of) HSCT, dialysis, Gram-negative bacteremia, severe bacterial infection, mild bacterial infection, local viral infection, disseminated viral infection, fungal infection.
Gram-negative bacteremia	abdominal surgery, diabetes mellitus type 2, atherosclerosis, (timing of) HSCT, dialysis, Gram-positive bacteremia, mild bacterial infection, severe bacterial infection, fungal infection.
local viral infection	thoracic surgery, abdominal surgery, diabetes mellitus type 2, atherosclerosis, carcinoma, radiotherapy, (timing of) HSCT, dialysis, Gram-positive bacteremia, severe bacterial infection, mild bacterial infection.
disseminated viral infection	thoracic surgery, abdominal surgery, diabetes mellitus type 2, atherosclerosis, COPD, splenectomy in past or during risk period, carcinoma, (timing of) HSCT, (acute or chronic) graft versus host disease, Gram-positive bacteremia, Gram-negative bacteremia, mild bacterial infection, fungal infection.
fungal infection	thoracic surgery, abdominal surgery, diabetes mellitus type 2, atherosclerosis, carcinoma, (timing of) HSCT, dialysis, Gram-positive bacteremia, Gram-negative bacteremia, severe bacterial infection, disseminated viral infection.
candidemia	abdominal surgery, diabetes mellitus type 2, atherosclerosis, COPD, organ transplant, carcinoma, (timing of) HCT, dialysis, Gram-positive bacteremia, Gram-negative bacteremia, severe bacterial infection, mild bacterial infection, local viral infection, disseminated viral infection.

Table S2 Continued.

Determinant	Confounders
Aspergillus infection	thoracic surgery, abdominal surgery, atherosclerosis, radiotherapy, (timing of) HSCT, dialysis, Gram-positive bacteremia, Gram-negative bacteremia, severe bacterial infection, mild bacterial infection, local viral infection.

All types of infections were associated with the variables listed under 'all'. In addition, several other potential confounders were identified per determinant. Atherosclerosis = systemic or coronary atherosclerosis. Chemotherapy = medication under subcategory L01 within the Anatomical Therapeutic Chemical (ATC) classification index. COPD = chronic asthma bronchiale or chronic obstructive pulmonary disease. Dialysis = || hemodialysis, peritoneal dialysis, or continuous veno-venous hemofiltration needed for at least one day during the risk period. GFR ≤ 30 ml/min = glomerular filtration rate (GFR) below 30 ml/min during at least one week of the risk period (calculated according to the Modification of Diet in Renal Diseases (MDRD) equation). HSCT = hematopoietic stem cell transplant. Immunosuppressant medication = medication under subcategory H02 (corticosteroids) or L04 (other immunosuppressants) within the ATC classification index.

Table S3 Overview of imputed data per recorded variable.

Variable	Type of variable (C / D)	Missing, N (%)	Variable	Type of variable (C / D)	Missing, N (%)
Age	C	0 (0)	(mature) lymphoma	C	3 (0.2)
Gender	C	0 (0)	Carcinoma	C	7 (0.5)
(duration of) ICU admittance	C	4 (0.3)	Chemotherapy	C	8 (0.5)
Thoracic surgery	C	0 (0)	Radiotherapy	C	0 (0)
Abdominal surgery	C	0 (0)	HSCT (in past or during risk period)	C	0 (0)
Diabetes mellitus type 2	C	1 (0.1)	Use of immunosuppressants	C	20 (1.3)
Atherosclerosis	C	17 (1.1)	Leukopenia	C	41 (2.7)
COPD	C	20 (1.3)	Maximum leukocyte counts	D	41 (2.7)
GFR ≤ 30 ml/min	C	2 (0.1)	Maximum CRP values	D	343 (22.6)
Dialysis	C	0 (0)	Gram-positive bacteremia	C+D	10 (0.7)
Splenectomy (in past or during risk period)	C	0 (0)	Gram-negative bacteremia	C+D	0 (0)
Organ transplant	C	0 (0)	Severe bacterial infection	C+D	47 (3.1)
Liver failure	C	2 (0.1)	Mild bacterial infection	C+D	27 (1.8)
Acute leukemia	C	1 (0.1)	Local viral infection	C+D	9 (0.6)
Myelodysplastic syndrome	C	3 (0.2)	Disseminated viral infection	C+D	0 (0)
Multiple myeloma	C	0 (0)	Fungal infection	C+D	13 (0.9)
Myeloproliferative neoplasm	C	4 (0.3)			

C = confounder of any determinant; D = determinant.
Atherosclerosis = systemic or coronary atherosclerosis. Chemotherapy = medication under subcategory L01 within the Anatomical Therapeutic Chemical (ATC) classification index. COPD = chronic asthma bronchiale or chronic obstructive pulmonary disease. Dialysis = || hemodialysis, peritoneal dialysis, or continuous veno-venous hemofiltration needed for at least one day during the risk period. GFR ≤ 30 ml/min = glomerular filtration rate (GFR) below 30 ml/min during at least one week of the risk period (calculated according to the Modification of Diet in Renal Diseases (MDRD) equation). HSCT = hematopoietic stem cell transplant. Immunosuppressant medication = medication under subcategory H02 (corticosteroids) or L04 (other immunosuppressants) within the ATC classification index.

Table S4 Infections diagnosed during the alloimmunization risk period.

A Time period = alloimmunization risk period

	Cases (N=505)	Controls (N=1,010)	RR (CI) *
Maximum leukocyte counts (x10^9/L) †			
4-10	140	249	*ref*
10-15	118	242	0.88 (0.56-1.20)
15-20	91	164	0.99 (0.70-1.39)
20-30	71	152	0.83 (0.56-1.21)
>30	43	96	0.79 (0.51-1.22)
Maximum CRP values (mg/L) ‡			
≤30	69	163	*ref*
30-100	95	184	1.20 (0.80-1.79)
100-200	120	259	1.08 (0.73-1.60)
200-300	126	219	1.37 (0.91-2.05)
>300	95	185	1.22 (0.77-1.92)

B Time period = 1st week following the implicated transfusion

	Cases (N=505)	Controls (N=1,010)	RR (CI) *
Maximum leukocyte counts (x10^9/L) †			
4-10	151	266	*ref*
10-15	143	251	1.02 (0.73-1.43)
15-20	76	158	0.84 (0.56-1.24)
20-30	59	110	0.97 (0.64-1.48)
>30	21	53	0.67 (0.36-1.23)
Maximum CRP values (mg/L) ‡			
≤30	73	150	*ref*
30-100	129	251	1.07 (0.57-2.03)
100-200	141	292	1.01 (0.59-1.72)
200-300	110	202	1.14 (0.72-1.81)
>300	51	115	0.92 (0.50-1.69)

Leukocytosis and elevated CRP values A. at least once measured during the alloimmunization risk period and B. at least once measured during the week following the implicated transfusion. Both did not predict the risk of red cell alloimmunization.

* Adjusted for: number of transfused red cell units and hospital. † as referenced to maximum leucocyte counts within the normal range (i.e. 4-10x10^9L). ‡ as referenced to maximum CRP values ≤ 30 mg/L. RR = relative risk. CI = 95% confidence interval.