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

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TREATMENTS FOR HEMATOLOGICAL MALIGNANCIES IN CONTRAST TO THOSE FOR SOLID CANCERS ARE ASSOCIATED WITH REDUCED RED CELL ALLOIMMUNIZATION

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Abstract

Red cell alloimmunization may induce severe hemolytic side effects. Identification of risk modifying conditions will help tailor preventative strategies. This study aims to quantify the associations of hematological malignancies and solid cancers with red cell alloimmunization in patients receiving red cell transfusions.

We performed a nested multicenter case-control study in a source population of 24,063 patients receiving their first and subsequent red cell transfusions during an eight year follow-up period. Cases (N=505), defined as patients developing a first transfusion-induced red cell alloantibody, were each compared with two non-alloimmunized controls (N=1,010) who received a similar number of red cell units. Using multivariate logistic regression analyses, we evaluated the association of various malignancies and treatment regimens with alloimmunization during a delineated 5-week risk period.

The incidence of alloimmunization among patients with acute (myeloid or lymphoid) leukemia and mature (B or T cell) lymphoma was significantly reduced as compared to patients without these malignancies (adjusted relative risks (RR) with 95% confidence interval (CI) 0.36 (0.19-0.68) and 0.30 (CI 0.12-0.81)). Associations were primarily explained by immunosuppressive treatments (RR for (any type of) chemotherapy combined with immunotherapy 0.27, CI 0.09-0.83). Alloimmunization risks were similarly diminished in allogeneic or autologous stem cell transplanted patients (RR 0.34, CI 0.16-0.74), at least during the six months post-transplantation. Alloimmunization risks of patients with other hematological diseases, solid cancers, and their associated treatment regimens were similar to risks in the general transfused population.

Our findings suggest that, in contrast to malignancies in general, hemato-oncologic patients treated with dose-intensive regimens have strongly diminished red cell alloimmunization risks.

Introduction

Transfusion of red cells causes exposure to non-self antigens and, consequently, may induce alloantibody formation. Although prior alloimmunization necessitates the exclusive administration of donor blood being negative for the cognate antigen, accidental re-exposure may induce severe hemolytic transfusion reactions.[1, 2] Prevention of alloimmunization and its consequences is pursued by transfusing ABO/RhD compatible units to all red cell recipients. In addition, matching beyond those antigens is recommended for certain patients considered to be at high risk of alloimmunization due to repeated exposure, since the number of transfusions is strongly associated with the likelihood of alloimmunization.[3-5] As such, in several high-income countries, patients with hemoglobinopathies and with myelodysplastic syndrome (MDS), who often face regular transfusions over long periods of time, receive red cell units matched for the most immunogenic and clinical relevant antigens C, c, E, e, and K.[3, 4]

The ability of the recipient's immune system to evoke a humoral alloimmune response upon red cell alloantigen exposure is likely modulated by the recipient's clinical condition.[6-8] In this regard, while oncologic patients were suggested to have a similar alloimmunization risk as compared to the general transfused population,[9-11] some studies reported high alloimmunization incidences among MDS patients.[12, 13] Importantly, apart from the study of Sanz et al,[13] these reports did not take the cumulative red cell exposure into account, which is often considerable in the oncologic patient population and a main determinant of alloimmunization.[5] Hence, a possible influence of disease-specific features is largely unclear. In addition, various cancer types differ from one another in their intrinsic immunobiological characteristics as well as in the immunosuppressive nature of their treatments. Therefore, alloimmunization rates observed in a heterogeneous oncologic patient population likely cannot be extrapolated to specific diseases.

We here report the results of a nested case-control study quantifying the associations of various hematological malignancies and solid cancers with the risk of red cell alloimmunization in a cohort of red cell transfusion recipients.

Methods

Study design and setting

We performed a nested case-control study within a mainly Caucasian source population of patients receiving their first and subsequent red cell transfusion between 2005 and 2013 at one of six Dutch participating hospitals. All six hospitals treat patients with oncological diagnoses, including standard remission-induction chemotherapy for acute leukemia patients. Allogeneic hematopoietic stem cell transplantations (HSCTs) are performed in three, and autologous HSCTs in four of these sites.

Details on the source population including eligibility criteria, the study period per hospital, and our methods were previously published[5, 14] and are described in detail in chapter 3 of this thesis.

In short, cases were all patients who developed a first transfusion-induced alloantibody against: 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. For all cases, we assumed the last antigen mismatched transfusion preceding the first positive screen (the *´Nth´* transfusion) to likely have elicited alloimmunization and defined this as the implicated transfusion. If this last mismatched transfusion could not be identified due to incomplete donor typing, the last non-tested unit preceding the first positive screen was considered as the implicated transfusion. For each case, we then randomly sampled two non-alloimmunized controls on the precondition that these patients received at least *N* or more transfusions in the same hospital, hereby following an 'incidence-density sampling strategy'.[15] After marking the *Nth* transfusion in the two matched controls, we subsequently constructed a so-called 'alloimmunization risk period' in both the case and the two controls, which stretches from 30 days before to seven days after this *Nth* (implicated) transfusion (for further illustration, see chapter 3, Figure 1 of this thesis). Next, by consulting the hospitals' electronic laboratory information systems and the medical charts of all patients, we recorded the presence of various clinical conditions during this period.

The study protocol was approved by the Ethical Review Board in Leiden and by the board of each participating center.

Data acquisition and statistical analyses

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 (hemato-) oncological diagnoses and treatment modalities.

The associations of hematological malignancies and solid cancers with the development of red cell alloimmunization were evaluated using conditional logistic regression models. For crude relative risk (RR) calculations, we conditioned on the matched variables i.e. hospital and cumulative number of red cell units received. To control for additional confounders, we first identified covariates as possible confounders of a given determinant, based on their observed association with this determinant among the source population (i.e. the non-alloimmunized controls).[16] Such an association was defined as a ≥3% difference in covariate presence between controls exposed and controls not exposed to a given determinant. Covariates in the causal pathway between the determinant and the outcome were not considered as confounders.[16] Second, to address missing data on these confounders, we performed multiple imputation creating five imputed datasets. Predictor variables included: alloimmunization status, age, gender, number of transfusions received,

(types of) malignancies, chemo- and/or immunotherapy, radiotherapy, use of immunosuppressant medication, (timing of) allogeneic and/or autologous stem cell transplant, graft versus host disease, (types of) infection, (duration of) fever, (duration of) ICU admittance, (types of) surgery, diabetes mellitus type 1, diabetes mellitus type 2, atherosclerosis, liver cirrhosis, renal insufficiency with a GFR ≤ 30 ml/min, dialysis, minimum leukocyte counts, maximum leukocyte counts, and maximum CRP values.

Third, to also accurately control for confounders with rare prevalences, we estimated a probability score for each determinant using logistic regression with the potential confounders as predictors.[17] Finally, we evaluated the association of various types of malignancies and treatment modalities with red cell alloimmunization by entering the corresponding probability scores next to the matching variables into the logistic regression model with alloimmunization as the outcome.

We next assessed the association between (the degree of) leukopenia and red cell alloimmunization. Missing leukocyte counts were similarly multiply imputed (see below). Minimum leukocyte counts were subcategorized into 2-4, 1-2 and <1x 10^9/L and referenced to normal counts (4-10x10^9/L). Since the likelihood that a low leukocyte count has been recorded at least once increases with the number of measurements and thus with the duration of hospitalization, we repeated this analysis limited to leukocyte counts measured within the week following the implicated transfusion.

A possible association between leukopenia (i.e. leukocyte counts <4x10^9/L) and type of malignancy was evaluated using Pearson's chi-square test.

As we used an incidence-density sampling procedure for selecting controls,[15] we interpreted and present all odds ratios as RR with 95% confidence intervals (CI).

Malignancies and their treatments

We used internationally accepted response criteria to define the remission state of various hematologic malignancies.[18-22] Malignancies in complete remission during the alloimmunization risk period were considered as absent. The presence of minimal residual disease was not taken into account. All medication under subcategory L01 in the World Health Organization's ATC (Anatomic Therapeutic Chemical) classification index[23] was defined as chemotherapy, with the exception of agents in the pharmacological subgroup L01XC as these involve monoclonal antibodies. Within subgroup L01XC and L04AA, we defined rituximab, alemtuzumab, and anti-thymocyte globulin (ATG, rabbit or horse derived) as (anti-lymphocyte) immunotherapy.

Results

Among 54,347 newly-transfused patients, 24,063 met all study criteria. The majority of excluded patients were ineligible due to the absence of an antibody screen following a single transfusion episode (N=25,037).

First-formed red cell alloantibodies were identified in 505 patients (2.1%, table S1). Thirty-seven of those patients (7.3%), including 21/32 (65.6%) who formed anti-Lu^a, only received units for which testing of the cognate antigen had not been performed. As explained, we here assumed the last non-tested unit preceding the first positive screen to have elicited alloimmunization.

General and clinical characteristics of the 505 alloimmunized patients and their 1,010 matched control subjects are presented in Table 1.

Table 1 Patient characteristics during the alloimmunization risk period.

Characteristics	Cases (N=505)	Controls (N=1010)	Missing
General			
Men	237 (46.9)	568 (56.2)	
Age in years (median, IQR)	67.0 (55.0-75.9)	65.3 (51.6-75.1)	
Cumulative number of red cell units received (median, IQR)			
lifetime*	4 (2-8)	4 (2-8)	
during risk period	3 (2-6)	4 (2-8)	
Days transfused during risk period (median, IQR)	1 (1-3)	2 (1-3	
Men	237 (46.9)	568 (56.2)	
Age in years (median, IQR)	67.0 (55.0-75.9)	65.3 (51.6-75.1)	
Patient diagnoses			
Diabetes mellitus (type 1 or 2)	97 (19.2)	183 (18.1)	1
GFR ≤ 30 ml/min †	56 (11.1)	149 (14.8)	
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)	
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
Treatment interventions			
ICU admission	177 (36.5)	369 (35.0)	
Surgery	267 (52.9)	457 (45.2)	2
Organ transplant	4 (0.8)	23 (2.3)	

Table 1 Continued.

Characteristics	Cases (N=505)	Controls (N=1010)	Missing
Treatment interventions			
Dialysis (either chronic or acute) ||	31 (6.1)	98 (9.7)	
Immunosuppressant medication ¶	154 (30.9)	423 (42.4)	20
Chemotherapy **	66 (13.1)	224 (22.2)	6
Radiotherapy	15 (3.0)	37 (3.7)	
Stem cell transplant (autologous or allogeneic, in past or during risk period)	10 (2.0)	63 (6.2)	
Treatment related complications			
Leukopenia ††	102 (20.2)	313 31.0)	
Graft versus host disease	4 (0.8)	15 (1.5)	3
Infections			
bacterial	142 (29.3)	275 (28.7)	72
viral	15 (3.0)	38 (3.8)	9
fungal	12 (2.4)	44 (4.4)	13

Values are n (%), unless otherwise stated. Numbers of patients with unavailable data per variable are presented as missing. IQR = interquartile range.

* up until the first positive screen for cases and up until the last available (negative) screen for controls. † 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). ‡ systemic or coronary atherosclerosis. § chronic asthma bronchiale or chronic obstructive pulmonary disease. || hemodialysis, peritoneal dialysis, or continuous veno-venous hemofiltration needed for at least one day during the risk period. ¶ medication under subcategory H02 (corticosteroids) or L04 (other immunosuppressants) within the World Health Organization's Anatomical Therapeutic Chemical (ATC) classification index. ** medication under subcategory L01 in the ATC classification index with the exception of agents in the subgroup L01XC (monoclonal antibodies). †† at least once measured leukocyte counts below lower limit of normal.

Malignancies present during the alloimmunization risk period

A total of 606 patients (40.0%) had at least one type of malignancy: 270 had a hematological malignancy, and 338 a solid tumor (two patients presented with both types of malignancies). Table S2 presents types and subtypes of malignancies.

The presence of a malignancy could not be confirmed for 12 patients: four patients with a clinical condition suspected for a malignancy that was not further evaluated, four patients with a suspected malignancy in whom a malignancy was later confirmed, and four patients receiving treatment for a solid tumor for whom the remission status at the time of the risk period was unclear. These 12 patients were omitted from the corresponding analyses.

Table 2 Association between various malignancies and red cell alloimmunization.

	Cases (N=505)	Controls (N=1,010)	RR (CI) *	Adjusted RR (CI) †	Excluded from analysis
Hematologic malignancies					
Acute leukemia	14 (2.8)	74 (7.3)	0.31 (0.17-0.58)	0.36 (0.19-0.68)	1
myeloid	14 (2.8)	62 (6.1)	0.38 (0.20-0.71)	0.41 (0.22-0.79)	0
lymphoblastic ‡	0 (0)	12 (1.2)	0.00 (NC)	0.00 (NC)	1
Myelodysplastic syndrome §	18 (3.6)	46 (4.6)	0.76 (0.43-1.36)	0.75 (0.41-1.36)	2
Multiple myeloma	10 (2.0)	26 (2.6)	0.77 (0.36-1.62)	0.79 (0.36-1.71)	0
Myeloproliferative neoplasm ‖	9 (1.8)	29 (2.9)	0.62 (0.29-1.33)	0.64 (0.29-1.41)	0
Chronic lymphatic leukemia	5 (1.0)	7 (0.7)	1.45 (0.45-4.67)	1.20 (0.36-3.93)	0
Lymphoma ¶					
all	5 (1.0)	35 (3.5)	0.27 (0.10-0.69)	0.30 (0.12-0.81)	2
(mature) B cell lymphoma	4 (0.8)	28 (2.8)	0.27 (0.09-0.77)	0.30 (0.10-0.89)	2
T cell lymphoma	1 (0.2)	6 (0.6)	0.33 (0.04-2.75)	0.37 (0.04-3.15)	2
Non-hematologic malignancies					
Carcinoma	112 (22.3)	183 (18.2)	1.30 (0.99-1.70)	1.01 (0.75-1.37)	7
Other	12 (2.4)	31 (3.1)	0.77 (0.39-1.53)	0.83 (0.41-1.68)	1

Values are n (%). * Adjusted for the matched variables: number of transfused red cell units and hospital. † Additionally adjusted for other potential confounders (for details, see Table S3 en S4). ‡ acute lymphoblastic leukemia and acute lymphoblastic lymphoma. § six patients were diagnosed with a myelodysplastic syndrome in combination with another hemato-oncological disorder. ‖ including polycythemia vera, essential thrombocytosis, primary myelofibrosis, juvenile and chronic myelomonocytic leukemia. ¶ One patient was diagnosed with an undifferentiated mature lymphoma. NC = not computable.

Table S3 and table S4 present identified confounders per type of malignancy. Control patients with acute leukemia and lymphoma, as compared to control patients without these diseases, were younger and had less comorbidity, including renal insufficiency and presence of other malignancies. They received more frequently chemotherapy and immunosuppressant medication and had more frequently decreased leukocyte counts. The frequency of missing data per identified confounder was maximally 2.7%.

The association between types of malignancies and red cell alloimmunization

Table 2 presents number of cases and controls according to various types of malignancies. Acute leukemia was present in 14 cases (2.8%) as compared to 74 (7.3%) controls. The incidence of red cell alloimmunization in patients with acute (myeloid or lymphoblastic)

Table 3 Treatment modalities and red cell alloimmunization risks.

	Cases (N=505)	Controls (N=1,010)	RR (CI) *	Adjusted RR (CI) †	Excluded from analysis
Chemo- and/or immunotherapy					6
type					
none	437 (86.9)	782 (77.7)	*ref*	*ref*	
(only) chemotherapy ‡	61 (12.1)	180 (17.9)	0.57 (0.41-0.79)	0.86 (0.54-1.36)	
(only) immunotherapy §	1 (0.2)	4 (0.4)	0.57 (0.06-5.67)	0.62 (0.07-5.18)	
chemo- and immunotherapy	4 (0.8)	40 (4.0)	0.17 (0.06-0.48)	0.27 (0.09-0.83)	
HSCT					0
type					
autologous or allogeneic ||	10 (2.0)	64 (6.3)	0.29 (0.14-0.58)	0.34 (0.16-0.74)	
timing (months before implicated transfusion)					
none	495 (98.0)	946 (93.7)	*ref*	*ref*	
0-1	4 (0.8)	27 (2.7)	0.28 (0.09-0.81)	0.34 (0.11-1.07)	
>1-6	3 (0.6)	24 (2.4)	0.22 (0.06-0.75)	0.24 (0.07-0.86)	
>6	3 (0.6)	13 (1.3)	0.46 (0.13-1.70)	0.55 (0.14-2.09)	
Radiotherapy	15 (3.0)	39 (3.9)	0.78 (0.42-1.44)	0.75 (0.39-1.44)	0

Values are n (%). * Adjusted for the matched variables: number of transfused red cell units and hospital. † Additionally adjusted for other potential confounders (for details, see Table S4). ‡ all medication under subcategory L01 within the Anatomical Therapeutic Chemical (ATC) classification index with the exception of monoclonal antibodies. § monoclonal antibodies directed against B and/or T lymphocyte markers received by 49 patients (rituximab N=20, alemtuzumab N=5, and anti-thymocyte globulin N=25). || 10 patients received an allogeneic HSCT after an earlier autologous HSCT. HSCT = hematopoietic stem cell transplant (either autologous or allogeneic) received before or during the alloimmunization risk period.

leukemia and in patients with mature (B or T cell) lymphoma was reduced (adjusted RR 0.36 (CI 0.19-0.68) and 0.30 (CI 0.12-0.81), respectively). Conversely, patients with chronic lymphatic leukemia (CLL) showed a modest, albeit statistically non-significant, increased risk (adjusted RR 1.20, CI 0.36-3.93). No association between the other types of malignancies and red cell alloimmunization was observed, including MDS and solid malignancies. Similarly, subtypes of solid tumors were not associated to red cell alloimmunization, although some RRs presented with wide CIs (Table S5). As extensive matching recommendations have only been introduced since 2011 in the Netherlands,[3] only 1 of 64 patients (1.6%) with MDS received CcEe and K matched units.

Effects were similar in all six hospitals (data not shown).

The association between treatment modalities and red cell alloimmunization

A total of 290 patients received chemo- and/or (anti-lymphocyte) immunotherapy during the implicated risk period. Use of any type of chemotherapy without immunotherapy was not associated with red cell alloimmunization. However, when regimens included lymphocyte-targeted monoclonal antibodies the adjusted RR was 0.27 (CI 0.09-0.83) (table 3). Twenty-five of the 49 patients (51%) treated with monoclonal antibodies received ATG (with or without alemtuzumab), aiming *in vivo* depletion of T cells in the context of an allogeneic HSCT (N=21), aplastic anemia (N=3), or combined pancreas-kidney organ transplant (N=1).

Patients receiving chemotherapeutic agents for acute leukemia or lymphoma during the implicated risk period had substantially reduced alloimmunization incidences (RR 0.29 (0.14-0.60) and 0.08 (0.01-0.57), respectively). This risk reduction seemed not majorly further influenced by the time interval between the initial diagnosis and the risk period (data not shown). In contrast, non-treated patients with these disorders demonstrated risks comparable to the remainder of the patient population (Table 4). Sixty-two of the 74 treated patients (84%) with acute leukemia received induction therapy during the allo-immunization risk period. Analogous to acute leukemia and mature lymphoma, the 22 patients who received treatment for their MDS (including 13 patients receiving induction therapy and seven receiving hypomethylating agents), demonstrated a trend towards reduced alloimmunization incidences (RR 0.31 (CI 0.09-1.06), Table 4). Chemotherapy did not modulate risks in patients with other types of hematological malignancies or carcinoma (Table 4).

A total of 54 patients received radiotherapy (of any dose and frequency), including 10 patients who received total body irradiation in the setting of an allogeneic HSCT. Radiotherapy was not associated with red cell alloimmunization (Table 3).

Respectively 51, 13, and 10 patients underwent an allogeneic HSCT, an autologous HSCT, or both in the time course preceding or during the risk period. In 51 patients, a reduced-intensity allogeneic HSCT conditioning regimen was followed (including eight patients who received a double cord transplant), whilst 10 patients received a myeloablative conditioning regimen. Alloimmunization incidences were substantially decreased in (allogeneic or autologous) in these stem cell transplant recipients (RR 0.34, CI 0.16-0.74), at least during the first six months after transplantation (Table 3). Alloimmunization risks did not differ between recipients of an autologous or allogeneic HSCT (data not shown).

Lastly, the degree of leukopenia was strongly associated with diminished red cell alloimmunization (Table 5). Here, patients with leukocyte counts of $<1.0x10^9$/L demonstrated an adjusted RR of 0.33 (CI 0.20-0.55). Similar results were obtained when we restricted these analyses to leucocyte counts determined within the week following the implicated transfusion (Table 5). The degree of leukopenia was associated with the type of malignancy and the receipt of chemotherapy. In this regard, minimum leukocyte counts of $<1.0x10^9$/L

Table 4 Chemotherapy and red cell alloimmunization risks.

Type of malignancy	Chemotherapy	Cases (N=505)	Controls (N=1,010)	RR (CI) *	Adjusted RR (CI) †
Acute leukemia					
-		489	931	*ref*	*ref*
+	-	4	10	0.77 (0.22-2.66)	0.88 (0.25-3.09)
+	+	10	64	0.25 (0.12-0.51)	0.29 (0.14-0.60)
Myelodysplastic syndrome					
-		484	959	*ref*	*ref*
+	-	15	28	1.06 (0.54-2.07)	1.04 (0.52-2.06)
+	+	3	18	0.32 (0.09-1.12)	0.31 (0.09-1.06)
Multiple myeloma					
-		493	981	*ref*	*ref*
+	-	4	7	1.14 (0.32-4.06)	1.19 (0.33-4.34)
+	+	6	18	0.67 (0.26-1.72)	0.70 (0.27-1.82)
Myeloproliferative neoplasm					
-		494	977	*ref*	*ref*
+	-	3	13	0.46 (0.13-1.63)	0.48 (0.13-1.73)
+	+	6	16	0.75 (0.29-1.95)	0.79 (0.30-2.09)
Chronic lymphatic leukemia					
-		499	999	*ref*	*ref*
+	-	1	3	0.49 (0.05-4.85)	0.67 (0.07-6.47)
+	+	3	4	1.27 (0.27-6.01)	1.53 (0.33-7.11)
Lymphoma					
-		498	969	*ref*	*ref*
+	-	4	7	1.08 (0.31-3.76)	1.26 (0.35-4.51)
+	+	1	28	0.07 (0.01-0.49)	0.08 (0.01-0.57)
Carcinoma					
-		390	821	*ref*	*ref*
+	-	85	141	1.28 (0.95-1.73)	0.99 (0.71-1.38)
+	+	26	39	1.40 (0.84-2.35)	1.14 (0.67-1.94)

+ = present; - = absent. Only numbers of patients for whom the presence or absence of a given malignancy and the use of chemotherapy during the alloimmunization risk period could be determined are presented. * Adjusted for the matched variables: number of transfused red cell units and hospital. † Additionally adjusted for other potential confounders (for details, see Table S4).

Table 5 Leukopenia and red cell alloimmunization risks.

Minimum leukocyte counts (x10^9/L) during:	Cases (N=505)	Controls (N=1,010)	RR (CI) *	Adjusted RR (CI) †
Alloimmunization risk period ‡				
4-10	307	524	*ref*	*ref*
2-<4	61	128	0.82 (0.58-1.15)	0.87 (0.61-1.24)
1-<2	14	43	0.52 (0.27-0.99)	0.59 (0.31-1.13)
<1	26	142	0.27 (0.17-0.44)	0.33 (0.20-0.55)
≤1 week following implicated transfusion				
4-10	273	485	*ref*	*ref*
2-<4	44	107	0.72 (0.47-1.10)	0.80 (0.52-1.23)
1-<2	15	41	0.60 (0.30-1.23)	0.75 (0.36-1.58)
<1	19	119	0.24 (0.13-0.44)	0.34 (0.17-0.66)

Minimum leukocyte counts as measured during the alloimmunization risk period and as measured during the week following the implicated transfusion. Values are n (%). Cumulative numbers of presented cases and controls do not necessarily equal the total number of cases and controls, as patients with leukocytosis are not presented. * Adjusted for the matched variables: number of transfused red cell units and hospital. † Additionally adjusted for other potential confounders (for details, see Table S4). ‡ p = 0.02 for trend analysis.

were observed in respectively 66.2%, 75.9%, and 13.8% of patients with acute leukemia, lymphoma, and carcinoma receiving chemotherapy during the risk period (p<0.0001 for carcinoma versus acute leukemia and for carcinoma versus lymphoma).

Discussion

In this nested case-control study, we evaluated whether patients diagnosed with hematological malignancies and solid cancers differed in their risk to form red cell alloantibodies as compared to the general transfused patient population. Patients treated for acute leukemia (either of myeloid or lymphoblastic origin) and patients with mature (B or T cell) lymphomas demonstrated a 3-fold decreased incidence of clinically relevant alloantibodies against red cell alloantigens. In contrast, alloimmunization incidences among patients treated for other hematological malignancies or solid tumors were similar to those among the non-malignant patient population.

Although earlier reports only observed similar or even increased red cell alloimmunization frequencies in the oncologic patient population,[9-11] these prevalence-based studies did not adjust for the substantial number of transfusions these patients usually receive. However, the cumulative transfusion dose is a well-known important determinant

of alloimmunization.[5] Consequently, the observed positive associations might have been completely due to a rather intensive red cell transfusion support that is generally needed in the treatment of certain malignancies rather than to disease-specific characteristics itself. Finally, no studies so far compared specific oncologic diseases for alloimmunization risks with one another.

Our findings suggest that especially the dose-intensive immunosuppressive therapy influences alloimmunization. This seems biologically plausible. Several classical cytotoxic agents frequently used in the treatment of acute leukemia and lymphoma, including cyclophosphamide, purine nucleoside analogs, and anthracyclines, are known to induce prolonged (mainly naive) $CD4^+$ T cell and B cell depletion.[24-27] Moreover, chemotherapeutic regimens often include corticosteroids, a class of immunosuppressants which we earlier reported for to protect against red cell alloimmunization.[8] Significantly reduced red cell alloimmunization incidences were also found in patients receiving anti-lymphocyte targeted agents (i.e. ATG, alemtuzumab, and rituximab). ATG is well known for its strong and prolonged T cell depleting effects.[28, 29] Additionally, ATG preparations contain antibodies against several B and even plasma cell-specific markers.[29, 30] In agreement, eradication of B cells by rituximab has been shown to coincide with impaired primary as well as recall vaccine responses.[31-34] Finally, we observed profoundly lower alloimmunization rates in the setting of an (either autologous or allogeneic) HSCT, which appeared to be sustained at least during the first six months after transplantation. Even though we cannot fully exclude the eight alloimmunizations following an allogeneic HSCT to have been elicited by donor-recipient red cell antigen mismatches (in addition to exposure via transfusion), these findings are consistent with previous studies reporting anti-D formation to be rare in RhD-negative HSCT recipients exposed to RhD.[35-37] Depending on age-associated thymic functioning, type of stem cell harvest, and intensity of T cell depletion strategies, reconstitution of adaptive immune cells generally takes up to six to 12 months following HSCT,[38-43] whilst humoral immunity may continue to be deficient, even after several years.[44, 45]

Although treatment-induced immunosuppression seems the principal explanation of our observations, other non-measured factors associated with receiving treatment (e.g. co-morbidities and disease stage) might have interacted with disease-specific effects on the immune response. Hence, we cannot exclude part of the observed effects to be directly related to the diseases themselves, i.e. induction of an immunosuppressive but tumor tolerant state via host immune evasion mechanisms of malignant cells.[46-49]

Furthermore, as patients received a large diversity of chemotherapeutic regimens at varying periods preceding the alloimmunization risk period, we were unable to reliably conclude whether and to what extent patients in complete remission of their treated malignancy should be considered as significantly immunosuppressed. As such, our presented RRs might underestimate true effects and our results do not preclude these patients to have a diminished red cell alloimmunization risk.

In contrast to some other studies,[12, 13] our incidence-based analysis did not demonstrate an enhanced alloimmunization susceptibility with a diagnosis of MDS. However, and similar to intensively treated patients with acute leukemia and mature lymphoma, patients who received treatment for their MDS tended to show reduced alloimmunization incidences. Consequently, the decision to transfuse extended donor-matched products to this patient population should not be based on the MDS diagnosis itself, but on other factors associated to an increased alloimmune response e.g. a high transfusion burden.

Finally, the alloimmunization RR in patients with chronic lymphatic leukemia (CLL) independent of their treatment seemed increased as compared to lymphoma patients, although we acknowledge that the number of CLL patients in the current study is insufficient to confirm such a hypothesis. Yet, CLL is characterized by profound immune disturbances including non-clonal formation of IgG auto-antibodies directed against blood cell antigens.[50-52] A disturbance of the normal regulatory potential by the disease has been implicated in these observations. Seemingly in contrast with these findings, antimicrobial vaccination responses are often compromised in CLL patients.[53]

Some final comments regarding our methods seem appropriate.

First, the use of an incidence-density sampling strategy guaranteed that controls were exposed to at least the same amount of red cell units as their matched cases.[15, 54] Given this adjustment for cumulative number of red cell exposures, our RRs reflect relative risks independent of exposures. Our defined alloimmunization risk period specifically functioned to comprehensively study the influential effect of conditions present around the time of red cell exposure. As the immunosuppressive effects of various treatment regimens only slowly extinguish, we preferred a relatively long risk period to precede the implicated transfusion.

Second, our strategies do not fully guarantee the exclusion of all boosting events. Actual 'lag periods' i.e. the time needed before antibody levels become detectable after primary antigen encounter, are currently unknown and may even differ per antigen. Regarding our chosen lag period of seven days, we thus cannot fully exclude to have included patients whose antibody titers became undetectable over time and demonstrated recall responses rapidly upon re-exposure to the alloantigen. However, 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 change RRs (data not shown). We therefore believe the eventual bias due to our choice of the lag period to be small.

Third, no associations of other than the above mentioned hematological malignancies and specific types of solid malignancies with red cell alloimmunization was observed, although the low numbers of some of these subgroups and the accordingly wide CIs per

RR prevent firm conclusions. A substantially larger study or a meta-analysis of similar studies is needed to assess whether these malignancies are truly indeed not associated to red cell alloimmunization. Also, due to incomplete remission evaluations available during the alloimmunization risk period, we were unable to assess whether the disease stage itself is associated to cell alloimmunization.

Finally, since patients treated with chemotherapy received a diversity of chemotherapeutic agents and combinations, as well as varying dose intensities, we were not able to quantify risks per single agent.

In conclusion, red cell alloimmunization risks are significantly reduced in patients treated for acute leukemia and mature lymphomas, as well as in recipients of an (autologous or allogeneic) HSCT. These diminished immune responses most likely reflect the intensity of treatment-associated immunosuppression. In contrast, alloimmunization risks in patients with other hematologic diseases and in patients with solid cancers are similar to those in the general, non-oncologic transfused patient population. These findings clearly indicate that, in addition to cumulative red cell exposure, disease-specific conditions should be taken into account when considering the risk of red cell alloimmunization, hereby ultimately aiming to select those who benefit most from extended matched red cell transfusions.

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Supplementary Material

Table S1 Specificity and distribution of first-formed red cell alloantibodies according to the presence and type of malignancy.

Alloantibody specificity	All patients, N (%)	Acute leukemia or mature lymphoma, N (%)	Carcinoma (%)
anti-C	23 (4.0)	0 (0)	4 (3.1)
anti-c	41 (7.2)	0 (0)	6 (4.7)
anti-E	185 (32.3)	4 (20.0)	43 (33.3)
anti-e	5 (0.9)	0 (0)	1 (0.8)
anti-K	126 (22.0)	3 (15.0)	32 (24.8)
anti-C^w	19 (3.3)	1 (5.0)	4 (3.1)
anti-Fy^a	31 (5.4)	0 (0)	3 (2.3)
anti-Fy^b	5 (0.9)	0 (0)	1 (0.8)
anti-Jk^a	54 (9.4)	3 (15.0)	17 (13.2)
anti-Jk^b	7 (1.2)	2 (10.0)	0 (0)
anti-Le^a	7 (1.2)	2 (10.0)	2 (1.5)
anti-Le^b	3 (0.5)	0 (0)	1 (0.8)
anti-Lu^a	32 (5.6)	3 (15.0)	9 (7.0)
anti-Lu^b	0 (0)	0 (0)	0 (0)
anti-M	22 (3.8)	2 (10.0)	3 (2.3)
anti-N	1 (0.2)	0 (0)	0 (0)
anti-S	12 (2.1)	0 (0)	3 (2.3)
anti-s	0 (0)	0 (0)	0 (0)
All antibodies	573	20	129
(possibly) natural occurring *	268 (46.7)	12 (60.0)	62 (48.1)
generally not inducing hemoysis†	55 (9.6)	5 (25.0)	12 (9.3)
N patients	505	19	112
N patients with ≥ 2 first-time alloantibodies	63 (12.5)	1 (5.3)	15 (13.4)

* including: anti-E, anti-C^w, anti-Le^a, anti-Le^b, anti-Lu^a, and anti-M. † Including: anti-Lu^a, anti-M and anti-N. The distribution of (possibly) natural occurring antibodies did not significantly differ between patients with acute leukemia or mature lymphoma as compared to the remaining of the study population, including patients with carcinoma (p=0.09, chi square test). In contrast, the frequency of non-hemolytic alloantibodies was higher in alloimmunized patients with acute leukemia or mature lymphoma as compared to the remaining of the immunized population (p=0.03). However, this did not affect conclusions presented in table 2 (data not shown).

Table S2 Categories and types of malignancies present during the alloimmunization risk period.

Hematologic malignancies	**N**
Diagnosed in *N* patients	270
Acute leukemia	88
myeloid (AML)	76
lymphoblastic (ALL) *	12
Myelodysplastic syndrome	63
Multiple myeloma	36
Myeloproliferative neoplasm	38
Chronic lymphatic leukemia	12
Lymphoma	40
(mature) B cell lymphoma †	32
T cell lymphoma ‡	7
undifferentiated	1

Other	**N**
Diagnosed in *N* patients	43
Germ cell tumors	4
Melanoma	1
Neuro-endocrine tumors	3
Stromal and mesenchymal neoplasms	35

Carcinomas	**N**
Diagnosed in *N* patients	295
Adrenal	2
Bile tract	2
Breast	21
Cervix, endometrial	14
Colorectal	71
Duodenal, stomach	15
Esophagus	11
Head and neck	17
Hepatic cell	6
Lung §	41
Ovarian	19
Pancreatic	7
Prostate	21
Renal cell	20
Squamous cell	3
Unknown primary origin ‖	3
Urothelial	20
Vaginal, vulvar	2
Other	1

Cumulative numbers of types of malignancies per category may exceed the number of patients per category, as some patients were diagnosed with two malignant diseases.

* acute lymphoblastic leukemia and acute lymphoblastic lymphoma. † of which: 6 patients with Burkitt lymphoma, 11 with diffuse large B cell lymphoma, 5 with follicular lymphoma, 1 with hairy cell lymphoma, 4 with Hodgkin lymphoma, 3 with mantle cell lymphoma, 1 with low-grade B cell lymphoma not otherwise specified, and 1 with lymphoplasmacytic lymphoma. ‡ of which: 3 patients with anaplastic T cell lymphoma, 1 with mycosis fungoides, and 3 with peripheral T cell lymphoma not otherwise specified. One patient was diagnosed with an undifferentiated mature lymphoma. § of which 37 patients with non-small cell lung carcinoma and 4 with small cell lung carcinoma. ‖ of which: 2 patients with adenocarcinoma with unknown primary and 1 with squamous cell carcinoma with unknown primary.

Table S3 Characteristics of 1,010 non-alloimmunizated controls during the alloimmunization risk period according to the presence and type of malignancy.

Characteristics	Acute leukemia		Lymphoma		Carcinoma	
	present (N=74)	not present (N=935)	present (N=35)	not present (N=973)	present (N=183)	not present (N=822)
General						
Men	46 (62.2)	522 (55.8)	25 (71.4)	542 (55.7)	102 (55.7)	463 (56.3)
Age in years (median, IQR)	55.1 (37.6-65.5)	66.2 (52.5-75.8)	57.3 (34.3-67.1)	65.5 (52.0-75.4)	67.7 (58.5-75.9)	64.0 (49.3-74.9)
Transfused in university hospitals	42 (56.8)	421 (45.0)	20 (57.1)	444 (45.6)	62 (33.9)	400 (48.9)
Cumulative (lifetime) number of red cell units received (median, IQR)	6.0 (3.0-12.5)	4 (2-8)	3.0 (2.0-7.0)	4.0 (2.0-8.0)	3.0 (2.0-6.0)	4.0 (2.0-8.0)
Cumulative number of red cell units during risk period (median, IQR)	7 (4-11)	4 (2-8)	3 (2-6)	4 (2-8)	3 (2-6)	5 (3-9)
Days transfused during risk period (median, IQR)	3 (2-5)	2 (1-3)	2 (1-3)	2 (1-3)	2 (1-2)	2 (1-3)
Patient diagnoses						
Diabetes mellitus type 1	0 (0)	7 (0.87	1 (2.9)	6 (0.6)	1 (0.5)	6 (0.7)
Diabetes mellitus type 2	7 (9.5)	169 (18.1)	2 (5.7)	173 (17.8)	31 (16.9)	145 (17.6)
GFR ≤ 30 ml/min *	3 (4.1)	146 (15.6)	3 (8.6)	146 (15.0)	21 (11.5)	128 (15.6)
Atherosclerosis †	7 (9.5)	307 (32.8)	1 (2.9)	312 (32.1)	44 (24.0)	269 (32.7)
Chronic obstructive airway disease ‡	3 (4.1)	86 (9.2)	2 (5.7)	86 (8.8)	14 (7.7)	75 (9.1)
Splenectomy (in past or during risk period)	0 (0)	19 (2.0)	0 (0)	19 (2.0)	3 (1.6)	16 (1.9)
Liver cirrhosis	0 (0)	24 (2.6)	0 (0)	24 (2.5)	4 (2.2)	20 (2.4)
Malignancies						
acute leukemia	n.a.	n.a.	0 (0)	74 (7.6)	0 (0)	74 (9.0)
myelodysplastic syndrome	3 (4.1)	43 (4.6)	1 (2.9)	45 (4.6)	0 (0)	46 (5.6)
multiple myeloma	0 (0)	26 (2.8)	0 (0)	26 (2.7)	0 (0)	26 (3.2)
myeloproliferative neoplasm	0 (0)	29 (3.1)	0 (0)	29 (3.0)	0 (0)	29 (3.5)
chronic lymphocytic leukemia	0 (0)	7 (0.8)	0 (0)	7 (0.7)	0 (0)	7 (0.9)
lymphoma	0 (0)	35 (3.7)	n.a.	n.a.	1 (0.5)	34 (4.1)
carcinoma	0 (0)	183 (19.6)	1 (2.9)	182 (18.7)	n.a.	n.a.

Table S3 Continued.

Characteristics	Acute leukemia		Lymphoma		Carcinoma	
	present (N=74)	not present (N=935)	present (N=35)	not present (N=973)	present (N=183)	not present (N=822)
Treatment interventions						
ICU admission	5 (6.8)	364 (38.9)	4 (11.4)	364 (37.4)	51 (27.9)	318 (38.7)
days at ICU (median, IQR)	0 (0-0)	0 (0-4.5)	0 (0-0)	0 (0-4)	0 (0-1)	0 (0-5)
Surgery						
thoracic including CABG	0 (0)	144 (15.4)	0 (0)	143 (14.7)	13 (7.1)	131 (15.9)
abdominal	1 (1.4)	180 (19.3)	0 (0)	181 (18.6)	55 (30.1)	126 (15.3)
back or spinal cord	0 (0)	11 (1.2)	0 (0)	11 (1.1)	1 (0.5)	10 (1.2)
Organ transplant	0 (0)	23 (2.5)	0 (0)	23 (2.4)	0 (0)	23 (2.8)
Dialysis (either chronic or acute) §	1 (1.4)	97 (10.4)	1 (2.9)	97 (10.0)	8 (4.4)	90 (10.9)
Immunosuppressant medication ‖	33 (44.6)	389 (41.6)	26 (74.3)	395 (40.6)	64 (35.0)	358 (43.6)
Chemotherapy ¶	64 (86.5)	155 (16.6)	28 (80.0)	191 (19.6)	39 (21.3)	180 (21.9)
Radiotherapy	0 (0)	39 (4.2)	4 (1.1)	35 (3.6)	21 (11.5)	18 (2.2)
HSCT (autologous or allogeneic, in past or during risk period) **	1 (1.4)	63 (6.7)	6 (17.1)	58 (6.0)	0 (0)	64 (7.8)
Treatment related complications						
Infections						
severe bacterial	4 (5.4)	165 (17.6)	2 (5.7)	166 (17.1)	33 (18.0)	135 (16.4)
Gram-negative bacteremia ††	6 (8.1)	38 (4.1)	2 (2.9)	42 (4.3)	9 (4.9)	35 (4.3)
disseminated viral ‡‡	0 (0)	20 (2.1)	2 (2.9)	18 (1.8)	0 (0)	20 (2.4)
Leukopenia §§	56 (75.7)	251 (26.8)	28 (80.0)	278 (28.6)	41 (22.4)	266 (32.3)

Values are n (%), unless otherwise stated. IQR = interquartile range. n.a. = not applicable. The presence of acute leukemia, mature lymphoma, and carcinoma could not be determined for one, two, and five control patients, respectively. * 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). † systemic or coronary atherosclerosis. ‡ chronic asthma bronchiale or chronic obstructive pulmonary disease. § hemodialysis, peritoneal dialysis, or continuous veno-venous hemofiltration needed for at least one day during the risk period. ‖ medication under subcategory H02 (corticosteroids) or L04 (other immunosuppressants) within the World Health Organization's Anatomical Therapeutic Chemical (ATC) classification index. ¶ medication under subcategory L01 in the ATC classification index, with the exception of agents in the subgroup L01XC (monoclonal antibodies) ** hematopoietic stem cell transplant. †† abscesses, cardiac infections, infected foreign material, intra-abdominal infections, lower respiratory tract infections, meningitis, osteomyelitis, soft tissue infections, spondylodiscitis, upper urinary tract infections. ‡‡ viremia and varicella zoster infections. §§ at least once measured leucocyte counts below lower limit of normal.

Table S4 Subset of variables identified as confounders per determinant for alloimmunization.

Determinant	Confounders
All	Age, gender, (duration of) ICU admittance, thoracic surgery, atherosclerosis, GFR ≤ 30 ml/min, dialysis.
Acute leukemia	Idem as under 'all', plus: abdominal surgery, DM2, COPD, MPN, lymphoma, carcinoma
Myelodysplastic syndrome	Idem as under 'all', plus: abdominal surgery, DM2.
Multiple myeloma	Idem as under 'all', plus: abdominal surgery, DM2, acute leukemia, lymphoma, carcinoma.
Myeloproliferative neoplasm	Idem as under 'all', plus: abdominal surgery, COPD, lymphoma, carcinoma.
Chronic lymphocytic leukemia	Idem as under 'all', plus: abdominal surgery, DM2, COPD, acute leukemia, MDS, lymphoma, carcinoma.
Lymphoma	Idem as under 'all', plus: abdominal surgery, DM2, COPD, acute leukemia, MPN, carcinoma.
Carcinoma	Idem as under 'all', plus: abdominal surgery, acute leukemia, MDS, MM, MPN, lymphoma.
Other malignancies	Idem as under 'all', plus: abdominal surgery, back or spinal surgery, splenectomy in past or during risk period, acute leukemia, MDS, lymphoma, carcinoma.
Chemo-/immunotherapy	Idem as under 'all', plus: abdominal surgery, DM2, COPD, immunosuppressant medication, acute leukemia, MDS, MM, MPN, lymphoma, carcinoma.
Radiotherapy	Idem as under 'all', plus: DM2, COPD, acute leukemia, MM, lymphoma, chemo-/immunotherapy, carcinoma.
Autologous stem cell transplant	Idem as under 'all', plus: abdominal surgery, DM2, COPD, acute leukemia, MM, MPN, lymphoma, carcinoma.
Allogeneic stem cell transplant	Idem as under 'all', plus: abdominal surgery, DM2, COPD, acute leukemia, MM, MPN, lymphoma, carcinoma, (timing of previous) autologous HSCT.
(degree of) leukopenia	Idem as under 'all', plus: abdominal surgery, DM2, COPD, immunosuppressant medication, acute leukemia, MDS, MM, carcinoma, chemo-/immunotherapy, radiotherapy, (timing of) HSCT.

All determinants were associated with the variables listed under 'all'. In addition to these, several other potential confounders were identified per determinant.
Atherosclerosis = systemic or coronary atherosclerosis. GFR = 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). Dialysis = hemodialysis, peritoneal dialysis, or continuous veno-venous hemofiltration needed for at least one day during the risk period. DM2 = diabetes mellitus type 2. COPD = chronic asthma bronchiale or chronic obstructive pulmonary disease. MDS = myelodysplastic syndrome. MPN = myeloproliferative neoplasm. MM = multiple myeloma. Immunosuppressant medication = medication under subcategory H02 (corticosteroids) or L04 (other immunosuppressants) within the Anatomical Therapeutic Chemical (ATC) classification index. Chemo-/immunotherapy = medication under subcategory L01 within the ATC index plus antithymocyte globulin. HSCT = hematopoietic stem cell transplant.

Table S5 Association between non-hematological malignancies and red cell alloimmunization according to specific type of malignancies.

	Cases (N=505)	Controls (N=1,010)	RR (CI) *	Adjusted RR (CI) †	Excluded from analysis
Carcinoma	112 (22.3)	183 (18.2)	1.30 (0.99-1.70)	1.01 (0.75-1.37)	7
Breast	8 (1.6)	13 (1.3)	1.30 (0.53-3.18)	1.02 (0.40-2.58)	0
Colorectal	24 (4.8)	47 (4.7)	1.08 (0.64-1.81)	0.86 (0.49-1.49)	0
Lung	17 (3.4)	24 (2.4)	1.47 (0.77-2.78)	1.22 (0.63-2.37)	0
Prostate	4 (0.8)	16 (1.6)	0.53 (0.17-1.61)	0.49 (0.16-1.53)	0
Renal cell	6 (1.2)	14 (1.4)	0.91 (0.35-2.41)	0.83 (0.31-2.23)	0
Urothelial	7 (1.4)	13 (1.3)	1.11 (0.44-2.84)	1.09 (0.41-2.89)	0
Other	12 (2.4)	31 (3.1)	0.77 (0.39-1.53)	0.83 (0.41-1.68)	1
Stromal and mesenchymal	9 (1.8)	26 (2.6)	0.69 (0.31-1.50)	0.74 (0.33-1.65)	1

Values are n (%). Only subtypes of solid tumors with at least 20 patients diagnosed are presented. * Adjusted for the matched variables: number of transfused red cell units and hospital. † Additionally adjusted for other potential confounders (for details, see Table S4).