Absence of the spleen and the occurrence of primary red cell alloimmunization in humans

With its unique anatomy and location amidst the circulatory system, the spleen allows an intimate contact between its resident cells and blood passing through the organ. Senescent and damaged red cells are primarily sequestered in the splenic red pulp and consumed by its macrophages.[1] Consequently, this route facilitates the presentation of non-self antigens of transfused red cells to splenic immune cells as a first and essential step in red cell alloimmunization. Indeed, the splenic microenvironment has been demonstrated to play a prominent role in red cell alloimmunization in mice.[2,3] Contrasting these animal studies, some observational studies in thalassemia patients suggested splenectomy to be associated with increased red cell alloimmunization,[4,5] while others did not find any association.[6,7]

In the study herein, we assessed the association between the anatomic absence of the spleen and (transfusion-related) red cell alloantibody induction in our multicenter case-control Risk Factors for Alloimmunization to Red Blood Cell Transfusion (R-FACT) study cohort. This cohort includes 505 alloimmunized cases and 1,010 non-alloimmunized matched controls among a primarily Caucasian source population of 24,063 patients receiving their first and subsequent red cell transfusions between January 2005 and December 2013 at one of six participating hospitals in the Netherlands, as described earlier.[8] A detailed description of our case-control cohort and the methodology used has been published recently.[9]

In summary, cases were identified as all patients who developed a first transfusion-induced alloantibody during the course of their transfusion history against the 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. Herein, we considered the last (documented or assumed) antigen mismatched transfusion preceding the first positive screen (i.e., 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. Based on an 'incidence density sampling strategy', for each identified case we randomly sampled two non-alloimmunized control subjects out of the source population, on the precondition that these controls had received at least an equivalent number of (lifetime) red cell transfusions in the same study center as the case. The *Nth* transfusion in these sampled controls, corresponding to the implicated transfusion of their matched cases, was then marked. Subsequently, we constructed a so-called 'alloimmunization risk period' in both cases and controls, stretching from 30 days before to seven days after this *Nth* (implicated) transfusion. Finally, we compared the presence of a history of splenectomy at the time of the alloimmunization risk period in cases and controls.

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

At the alloimmunization risk period, splenectomy had been performed in 20 patients, namely one case (0.2%) *versus* 19 controls (1.9%) (Table 1). In 12 patients, splenic injury was caused by severe trauma or complicated

Table 1. Demographics and splenectomy details of 19 non-alloimmunized and 1 alloimmunized splenectomized patients.

Patient	Age (years)/ sex	Allo-immunization	Indication for splenectomy
A	70/M	Yes	orthotopic liver transplantation complicated by splenic damage.
B	30/M	No	total pancreatectomy complicated by retroperitoneal hematoma and splenic infarction.
C	16/F	No	spontaneous splenic rupture shortly following post allogeneic stem cell transplantation.
D	39/M	No	severe trauma with intra-abdominal organ damage.
E	34/F	No	pregnancy complicated by rupture of a splenic artery aneurysm.
F	40/M	No	severe trauma with intra-abdominal organ damage.
G	74/F	No	resection of a large intra-abdominal liposarcoma, including splenectomy.
H	58/F	No	unilateral nephrectomy for renal cell carcinoma complicated by splenic damage.
I	72/F	No	resection of a large intra-abdominal liposarcoma.
J	55/M	No	*polycythemia vera* associated splenomegaly.
K	82/M	No	distal pancreatectomy with splenectomy.
L	46/M	No	severe trauma with intra-abdominal organ damage.
M	30/M	No	severe trauma with intra-abdominal organ damage.
N	63/M	No	pancreatic necrosis following a history of pancreaticojejunostomy.
O	49/M	No	severe trauma with intra-abdominal organ damage.
P	76/M	No	coronary artery bypass surgery complicated by an incarcerated inguinal hernia with secondary peritonitis and intra-abdominal hemorrhage.
Q	77/F	No	resection of a large intra-abdominal sarcoma.
R	67/F	No	adrenalectomy for metastasized adrenal carcinoma complicated by splenic damage.
S	75/M	No	unilateral nephrectomy for renal cell carcinoma complicated by severe intra-abdominal bleeding.
T	73/M	No	infective endocarditis with septic embolism and splenic abscesses.

Anti-M and anti-E were detected in patient A on days 23, 23 and 21 after the first allo-M and allo-E exposure, the splenectomy, and the implicated transfusion, respectively.

abdominal surgery, while no patient underwent a splenectomy in the context of an autoimmune disease. Sixteen of the splenectomized patients received their implicated (*Nth*) transfusion at or after splenectomy (median 0, range 0-3,612 days). In three other patients, splenectomy followed the implicated transfusion by 1-4 days. Consequently, in these patients immunization against the administered blood was considered as being modulated by the splenectomy. Subsequent red cell transfusions beyond splenectomy were received by all but two (patients L and N) controls (median 19 units; range 0-59, Table 2), with one control being further transfused beyond the study period. Red cell alloantibodies were not developed (data available up to April 2017).

Only one splenectomized patient developed alloantibodies (patient A). In this patient, anti-E and anti-M were simultaneously detected 23 days after a combined orthotopic liver transplantation and splenectomy. During, and following on from this surgery, he received 6 E-positive and at least 8 M-positive units. Using multivariate logistic regression analysis conditioning on the matched variables plus identified potential confounders (*Online Supplementary Table S1*), we estimated that splenectomized patients had a 20-fold reduced risk of alloimmunization as compared to patients lacking a history of splenectomy (adjusted relative risk (RR) 0.05, 95% confidence interval (CI) 0.01-0.55). Omitting patients L and N, who were not further exposed to red cell transfusions following splenectomy, did not change the RR (0.05 [95%CI 0.01-0.62]).

Since transfusions were administered both before and after splenectomy, the estimation of an alloimmunization risk from the time of splenectomy onwards should be related to both pre- and post-splenectomy red cell exposures. Based on an estimated number of 245 splenectomized patients within the entire source population, we calculated that 13 splenectomized patients, instead of only patient A, were expected to have developed alloantibodies had splenectomy not influenced alloimmunization (for calculations, see Table 2). We hereby assumed the red cell exposures of the 19 splenectomized controls to represent the red cell exposure pattern of all splenec-

Table 2. Illustration of expected *versus* observed numbers of alloimmunized patients within the splenectomized source population.

Patient	T1: number of red cell units received before splenectomy	T2: cumulative number of red cell units received up to last screen	pT1	pT2	*P*
A	0	19	0.000	0.063	0.063
B	0	2	0.000	0.016	0.016
C	15	30	0.061	0.084	0.023
D	0	11	0.000	0.051	0.051
E	0	16	0.000	0.063	0.063
F	0	8	0.000	0.037	0.037
G	0	31	0.000	0.084	0.084
H	0	19	0.000	0.063	0.063
I	4	31	0.027	0.084	0.057
J	0	34	0.000	0.089	0.089
K	0	19	0.000	0.063	0.063
L	2	2	0.016	0.016	0.000
M	0	10	0.000	0.047	0.047
N	21	21	0.067	0.067	0.000
O	0	59	0.000	0.104	0.104
P	30	53	0.084	0.104	0.019
Q	4	13	0.027	0.058	0.031
R	0	21	0.000	0.067	0.067
S	1	38	0.010	0.089	0.079
T	0	53	0.000	0.104	0.104
SUM					1.059

Step 1: Estimation of number of splenectomized patients within the source population. Among 14,901 patients from the Leiden University Medical Center, University Medical Center Utrecht and Jeroen Bosch Hospital 's Hertogenbosch, 155 patient with a documented history of splenectomy receiving red cell transfusions beyond their splenectomy were identified by searching their clinical files *via* information technology resources. None of these patients developed red cell antibodies. As these patients represent 62.0% of the entire source cohort, the total number of splenectomized patients within the source cohort will be approximately 245. Step 2: Comparison of expected *versus* observed number of alloimmunized patients within the splenectomized source population. Based on the cumulative number of red cell units received pre- and post-splenectomy, and reported cumulative incidences according to number of red cell units transfused,[8] the expected alloimmunization risk per splenectomized patient encountered from splenectomy onwards (Δp) can be deduced from the absolute risk at the time of splenectomy (pT1) and the risk at the time of last serological follow up (pT2). pT1 = the chance to have developed red cell alloantibodies following the number of red cell exposures at T1. pT2 = the chance to have developed red cell alloantibodies following the number of red cell exposures at T2. Δp= the chance to have developed red cell alloantibodies between T1 and T2 (i.e., following splenectomy). *P*-values were deduced from reported cumulative incidences according to number of red cell units transfused.[8] Consequently, had splenectomy not influenced alloimmunization, one would have expected 1.059 alloimmunizations per 20 splenectomized patients. This number corresponds to an estimated total of 13 alloimmunizations among the estimated 245 splenectomized patients (5.3%). As only one splenectomized patient within the source population developed alloantibodies, it seems conceivable that approximately 12 patients were protected from alloimmunization due to splenectomy, corresponding to a crude relative risk of 0.08.

tomized patients within the source population.

To the best of our knowledge, this is the first study in humans reporting red cell alloimmunization to be highly unlikely following splenectomy. Our observation underlines the spleen's function in protective adaptive immunity against non-self antigens present in the circulation, and corroborates with earlier studies in splenectomized mice. Even in the setting of poly(I:C) induced inflammation (a condition strongly linked with alloimmunization), murine red cell alloimmune responses were completely abrogated and were suggested to be due to a splenectomy induced impairment of $CD4^+$ T-cell priming and expansion.[2,3] Since T-cell priming requires efficient antigen presentation, it seems unsurprising that splenic conventional $CD11c^+$ dendritic cells have been strongly implicated in murine red cell alloimmunization.[10] In agreement with these findings, the splenic T cell subsets were shown to be pivotal for antibody production against both autologous and allogeneic platelet membrane antigens.[11]

Contrary to our results, thus far observational studies in patients with major thalassemia and sickle cell disease (a population not included in the current study) did not find any abrogation of red cell antibody development with splenectomy. Some even concluded that these patients were more prone to red cell alloimmunization.[4,5] Yet, hemoglobinopathy patients in need of splenectomy are often highly transfusion dependent, causing a prior high exposure related cumulative alloimmunization risk.[8] As such, exposure related confounding cannot be excluded as most of these studies did not correct for the cumulative exposure at the time of primary alloimmunization. Second, none reported the timing of alloimmunization to splenectomy nor the transfusion burden at the time of splenectomy, leaving the question of whether alloimmunization, or even only $CD4^+$ T-cell sensitization,[12] had not already occurred prior to splenectomy. With regard to the latter, alloimmunization following splenectomy could as such represent a T-cell dependent process and may explain why some hemoglobinopathy patients still develop alloantibodies despite the absence of the spleen. In addition, it is unknown how a functional deficiency of the spleen, as is known to be frequent in sickle cell disease patients, modulates red cell alloimmunization. As such, we argue that it is important to re-evaluate primary alloimmunization potentials in hemoglobinopathy patients with either anatomic or functional asplenia by carefully taking into account the above mentioned methodological issues, in order to elucidate the spleen's role in immunization against allogeneic blood cells in this specific patient population.

Concerning the anti-E and anti-M formed by the splenectomized patient A, we should first recognize that they might have developed independently of red cell exposure, i.e., as so-called "naturally occurring antibodies". Second, the induction of anti-M (if from the IgM class) might implicate a T cell-independent humoral immune response, for which the spleen is known to be essential.[13] Although an accessory spleen, present in over 10% of humans, was not identified *via* post-splenectomy CT scanning of the abdomen, some functional splenic tissue might have remained after splenectomy which mediated alloimmunization. Third, the specific combination of a donor liver transplant with splenectomy could have caused red cell alloimmunization *via* pre-primed lymphocytes derived from the donor's liver transplant (i.e., passenger lymphocyte syndrome). A similar mechanism has been reported in a patient developing nonhemolytic anti-M after multiorgan transplant.[14] Unfortunately, we could not retrieve the red cell antigenic phenotype of the liver donor to corroborate this hypothesis. Finally, we do not imply an absolute abolishment of red cell alloimmunization after splenectomy. Indeed, substantial evidence shows that at least a few asplenic patients are still capable of constructing a protective immune response following an unconjugated polysaccharide vaccination.[15] In addition, the absence of a functional spleen can, at least partly, be compensated by vaccines targeting a germinal center B cell response.[16] Yet, the non-intravenous route of vaccines and the common use of conjugates differ considerably from the administration of donor red cells, facilitating epitope presentation and efficient induction of T cell-dependent alloimmune responses in non-splenic lymphoid organs.

In conclusion, our findings suggest that splenectomy is strongly associated with protection from primary red cell alloimmunization in the general transfused patient population.

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