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

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CLINICAL DETERMINANTS OF RED CELL ALLOIMMUNIZATION

IMPLICATIONS FOR PREVENTATIVE ANTIGEN MATCHING STRATEGIES

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GENERAL INTRODUCTION

Red blood cells, their antigens, and alloimmunization

Red blood cell transfusions are a cornerstone for the management of patients with compromised hematopoiesis and for those losing large amounts of blood. Over 343,000 registered donors supplied over 427,000 red cell units in the Netherlands in 2015,[1] of which around 20% were transfused to patients with oncologic disease entities.[2]

Encountering allogeneic red blood cells e.g. by transfusion, pregnancy or organ transplant exposes one to polysaccharide and protein structures that may be different from the recipient's own structures and may therefore be recognized by the immune system. Different types of membrane-bound structures, such as lipids and (glyco)proteins anchored to the outer red cell membrane and participating in diverse cellular functions may be involved. Due to genetically determined interindividual variations, some of these surface markers are capable to induce antibody formation and hence, have been defined as red cell antigens.[3]

More than 300 of these inherited red cell antigens so far have been identified in humans,[4] and have been organized into 36 blood group systems.[5] Each blood group system represents the variation occurring in a single gene or in a cluster of two or more closely linked homologous genes.[3]

One of the most well-known (and probably the most complex) red cell antigens is Rhesus (Rh) D. A complete deletion of the *RHD* gene, present in 15% of the Caucasian population,[6] results in a complete absence of the approximately 30 kd D protein from the red cell surface.[7] In addition to a complete deletion, the *RHD* allele (and to a lesser degree the *RHCE* allele) can be subject to mutations that result into variances of the D polypeptide e.g. partial D and weak D expression.[8] Here, the D antigen is not completely absent, but lacks some common epitopes (partial D) or covers the red cell surface at a lower site density as compared to normal D (weak D). D variants are more common in people from African origin as compared to the Caucasian population.[9]

Contrasting D, blood group antigens within most other blood group systems result from single nucleotide polymorphisms (SNPs) in the allele, thereby not interfering with the antigen's expression, but resulting into aminoacid substitutions and consequently conformational changes of the protein. For example, the two major co-dominant alleles FY^a and FY^b differ from one another by one single nucleotide at position 125 (G vs A), resulting in a glycine or an aspartic acid amino acid at position 42 of the extracellular amino-terminal domain of the protein.[10]

As a result of the polymorphic nature of red cell antigens, encountering donor red cells expressing non-self antigens might provoke the recipient's immune system and induce an immune response towards these alloantigens. The final outcome of this immune response is the formation of alloantibodies. This process is called 'red cell allo-immunization' and forms the focus of this thesis.

Red blood cell alloimmunization: basic immunological principles

Although a full immunology review is beyond the scope of this thesis, some general concepts of a humoral immune response, and in particular the process of red cell allo-immunization, might help understand the here presented studies.

Antibody formation: a delicate interplay of innate and adaptive immunity

The immune system is typically divided into an innate and an adaptive immune system. Innate immunity refers to non-specific defense mechanisms that come into play immediately or within hours of a foreign antigen's appearance. As such, the innate immune system provides a first line of defense against common structures associated with microorganisms. In contrast, an adaptive immune response involves a more complex, antigen-specific response that is initiated only days following foreign antigen exposure. An adaptive immune response enables an immunological memory. As such, upon repeated contact with the antigen, the immune system can generate a faster and more magnified response.

The innate and adaptive immune system form no separate systems, but strongly cooperate with antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages. These APCs provide a crucial link between the two systems and serve as the sentinels of the immune system. Surveying the tissues and instructing the adaptive immune system in response to peripheral cues,[11-14] APCs with their pattern recognition receptors (PRPs) here recognize foreign chemical motifs (PAMPs) commonly present in non-mammalian organisms, as well as products released from damaged self-tissues (DAMPs). Both serve as 'danger signals', inducing APCs to mature and migrate to the peripheral lymphoid tissues (i.e. lymph nodes, the spleen and the liver). Here, they can prime antigen-naive cells of the adaptive immune system by presenting them processed discrete peptide fragments within the context of specific human leucocyte antigen (HLA) class I and II.[15,16] Interactions between costimulatory molecules expressed by the maturated APCs and their ligands on adaptive immune system cells are vital in this process.[17]

The adaptive immune system consists of T lymphocytes and B lymphocytes. Naive T cells fall into two large classes. CD8 cytotoxic T cells are critical for the defense against viruses and other intracellular pathogens. Via their T cell receptor (TCR), they recognize fragments of viral peptides in the context of HLA class I on the external surface of infected cells and are directly responsible for killing of these cells.[17] In contrast, $CD4^+$ T cells tightly orchestrate the behavior and activity of other immune cells by providing essential signals to these cells, but they do not have the intrinsic ability of pathogen clearance. Naive $CD4^+$ T cells are activated after interaction with antigenic peptides in HLA class II and subsequently differentiate into specific subtypes, the latter depending mainly on the cytokine milieu of

the microenvironment.[18,19] In contrast to cells of the innate immune system which can each recognize a wide diversity of pathogens, each cell of the adaptive immune system is restricted to respond to one specific antigen. As such, via a complex and elegant mechanism of gene rearrangements, a wide diversity in the antigen-receptor repertoire is generated, allowing accurate immune surveillance of the tissues.[17]

B lymphocytes are essential for the development of a humoral immune response. These cells are generated in the bone marrow after which they continue their development in the spleen further maturing into either follicular or marginal zone B lymphocytes.[20,21] Their primary function is to produce antibodies directed against foreign extracellular structures. B cells recognize foreign antigen via their unique B cell receptor (BCR), which, similarly to T cells, is generated via gene rearrangement processes hereby resulting into a wide range of antigen specificities to be represented in its repertoire. The BCR is a cell-surface immunoglobulin that has the same specificity as the secreted antibodies these cells eventually produce upon activation. When circulating matured follicular B cells access the follicular areas of peripheral lymphoid organs, they may recognize foreign antigen on APCs via their BCR, internalize, and subsequently present antigenic peptides in the context of HLA class II.

Upon antigen recognition, B cells proliferate and may rapidly differentiate into antibody-secreting plasmablast.[21] These cells are short lived. Hence, antibodies produced by these cells, often being from IgM class, are usually only shortly present after immunization.[16] A few of the antigen-engaged B cells will undergo further modifications in the germinal center of the secondary lymphoid organs (i.e. proliferation, somatic hypermutation, and immunoglobulin class switching), inducing the formation of highly effective plasmablasts that secrete high affinity antibodies of IgG class.[22-24] As this process of antibody formation requires accessory signals coming from primed $CD4^+$ T follicular lymphocytes (T_{FH}),[24] this route is called thymus-dependent B cell activation.

Contrasting thymus-dependent pathways, some antigens are capable to induce B cell proliferation in the absence of T cell help. These T-independent antigens for example comprise bacterial or viral structures such as lipopolysacharrides and specific DNA or RNA repeats.[25,26] In humans, splenic marginal zone B cells are important for this process.[20,27] Here, antigenic cross-linking of BCRs on B cells directly induces these B cells to become activated, maturate to plasmablasts, and secrete antigen-specific antibodies. T cell independent B cell responses usually do not induce immunoglobulin class switching and the process is thus characterized by IgM production. In the context of red cell alloimmunization, this route of antibody production applies to carbohydrate red cell antigenic structures e.g. antigens within the ABO and Lewis blood group systems.[28,29] In addition, alloantibodies against these antigens may occur "naturally" (i.e. lacking antigen exposure in the context of red cells) due to antigenic crosslinking with common bacterial polysaccharides. In this regard, isoagglutinins to the A and B antigens develop early during childhood, depending on the person's own bloodgroup, due to common exposure to

bacterial epitopes resembling these red cell antigens. As these anti-A and anti-B IgM antibodies are complement-binding, life-threatening intravascular hemolysis can occur upon an ABO mismatched red cell transfusion.

Antibody formation to red cell antigens

In contrast to most immunogens from microbial organisms being consumed and processed in lymph nodes, murine models have shown that senescent and damaged red cells are anatomically sequestered in the red pulp of the spleen and (to a lesser degree) in the liver.[30,31] Here, red pulp macrophages predominantly clear the majority of these red cells via phagocytosis.[30,32,33] Although less capable than DCs, macrophages are also involved in antigen presentation, hereby being able to activate antigen-specific B and T cells in case of red cell alloantigen exposure.

Contrasting the leading role of macrophages under steady state, in the presence of a pro-inflammatory stimuli such as the synthetic dsDNA poly(I:C),[34] CD11c+ DCs were identified as the primary contributors to splenic red cell consumption in mice.[31] Remarkably, plasmacytoid but not conventional DCs seemed responsible. DCs are potent antigen presenters and although conventional DCs are more equipped to this function than plasmacytoid DCs, both unconditionally require TLR-mediated activation for their functioning.[35] Consequently, it could be hypothesized that splenic DCs are critically responsible for the consistently reported enhanced red cell alloimmunization responses observed with poly(I:C) or CpG oligonucleotide induced inflammation.[30,36-38]

In consistence, substantially decreased antibody formation was observed in splenectomized mice as compared to non-splenectomized mice. This was shown to at least be the result of an impairment of $CD4^+$ T cell priming and expansion,[38] a process for which DCs again are pivotal. Consequently, antigen-engaged B cells will have been prevented from further differentiation into antibody-secreting cells, as they did not receive additional required signals. A possible restraint to B cell antigen-priming, as well as red cells potentially shunting away from an immunogenic organ towards a more tolerance inducing compartment (i.e. the liver)[39] are two other valid, though currently not evaluated, hypotheses.

Taken together, the spleen seems to play a pivotal role in red cell alloimmunization, at least in mice.

Red blood cell alloimmunization: the issue

The process of alloantibody formation in itself may not be harmful. Indeed, antibodies of several specificities are known to be seldomly of clinical relevance.[6] However, other alloantibodies potentially bind donor red cells expressing the allogeneic antigen and mediate accelerated destruction of these cells. The efficiency of this red cell clearance is

determined by the antibody's (sub)class, its affinity for the antigen, its complement activating capacity, the red cells' antigen density, and even the unique characteristics of the individual's Fcγ receptors on mononuclear cells.[16,40,41] Antibodies that have the potential to bind and activate complement may cause intravascular, often severe, red cell lysis by induction of membrane-attack complexes. In contrast, red cells opsonized with antibodies that lack complement binding are mainly removed in the spleen by cells of the mononuclear phagocyte system, while complement binding to red cells makes their clearance in the liver more likely.[16]

Acute, intravascular IgM and/or complement mediated hemolysis occurs within 24 hours of blood transfusion. ABO incompatible red cell transfusions in the presence of IgM antibodies directed against the A or B antigens are often the responsible cause.[3] Acute reactions may present with sudden onset of fever, flushing, hypotension, pain, dyspnea, hemoglobinuria, renal failure, and disseminated intravascular coagulation. As a result of current ABO/RhD matching and stringent antibody screening policies, these complement-mediated reactions have become relatively uncommon with an estimated frequency of 1 per 100,000 red cell units administered.[42] Despite, international hemovigilance reports have been documenting them for more than two decades as one of the main leading causes of transfusion-associated fatalities, with the major part of these events resulting from clerical errors.[43-46]

Delayed hemolytic transfusion reactions (DHTR) occur at a higher frequency as compared to acute reactions.[42] As antibody evanescence limits the ability to detect previous immunization by pretransfusion antibody screen, re-exposure to a given red cell antigen may evoke a secondary immune response in a patient previously sensitized to the antigen. Typically, antibodies directed against Rh, Kidd, and Duffy-system antigens are implicated in these reactions.[47,48] Symptoms may vary from only an asymptomatic positive direct antiglobin test (DAT) reaction to fulminant hemolysis resulting in severe anemia (i.e. delayed serological vs delayed hemolytic transfusion reaction). Difficulties in obtaining compatible blood or fear of further alloimmunization may in these cases even prevent receiving needed blood transfusions. Several cases of deceased alloimmunized sickle cell disease patients due to a DHTR have been reported.[49] In addition to inducing hemolysis of donor blood, anti-D among others is known for its capability to induce hemolytic disease of the fetus or newborn (HDFN) due to maternal immunization against the paternal antigens of the unborn child.[50,51]

Finally, and in addition to the above mentioned clinical complications, the substantial financial costs and logistical challenges brought about by red cell alloimmunization deserve emphasis. Determination of the specificity of the detected alloantibodies, often by complex and additional serologic work-up, and the difficulties in supplying sufficient compatible donor blood are unavailable consequences of alloimmunization once it has occurred.

Red blood cell alloimmunization: detection

Type and screen

Prior to their first transfusions, all patients are routinely typed twice for ABO and RhD and subsequently transfused with blood compatible for these antigens.[52] Typing of minor antigens is not performed on routine basis and depends on the policy described in (national) guidelines, which may be different for certain specific indications. Such indications involve specific diagnoses or clinical situations known to induce a chronic transfusion support or a higher chance of alloimmunization, e.g. previously immunized patients and patients with hemoglobinopathy.[52] In this regard, knowledge on which antigens are not expressed on the patient's red cells enables to select donor units that similarly lack expression of these antigens and thus will not elicit alloimmunization.

In addition to blood group typing, patients in the Netherlands are routinely screened for the presence of red cell alloantibodies within 72 hours prior to each red cell transfusion. Screening involves testing the patient's serum against a commercially available 3-red cell test panel, which combines expression of all clinically relevant antigens. Aiming to avoid false-negative test results, this 3-cell screening panel is required to homozygously express the antigens D, C, c, E, e, k, Fy^a, Fy^b, Jk^a, Jk^b, M, S and s, while K needs to be present minimally heterozygously.[52] The presence of C^w, Lu^a, Wr^a, and Kp^a is not mandatory[52] and hence, antibodies against these antigens might therefore not become detected by screening. The technique involves an indirect antiglobulin test (IAT) performed at 37°C. Here, the patient's serum is incubated with donor red cells. If present, alloantibodies from the patient's serum will bind to their cognate antigens expressed by the test red cells. As monomeric IgG antibodies cannot cross-link adjacent cells, IgG opsonized donor red cells will only agglutinate after adding a polyclonal mixture of anti-human IgG antibodies. This agglutination is visualized by a clump of red cells. When this screening test is positive, the antibody specificity is subsequently determined using the same technique with one or more extended panels of donor red cells of known phenotypes.[3,52]

For all patients with clinically relevant alloantibodies, neonates up till three months of age, patients who received a solid organ transplant as well as those who received an ABO incompatible allogeneic stem cell transplant, an additional cross match safeguard procedure is required to ascertain donor compatibility. This cross matching involves testing the patient's serum against the donor erythrocytes unit via the same IAT technique described above.

Genotyping

Although standard serological typing currently remains the gold standard, in the last few decades other techniques adding to the sensitivity of typing have been intensively sought for.

Compared to conventional serology, DNA-based methods are better suited to detect the presence of variant antigens.[53] For example, with serological typing, prophylactic D, C,

E, and K matching did not prevent sickle cell disease patients to develop a high rate of antibodies with Rh specificities even despite receiving blood from predominantly African-American donors (i.e. ethnically matched).[54,55] As of 1 May, 2016, an actively maintained database contains 45 red blood cell genes with together 1,744 alleles[4,56] and only the allelic sequence of some common nonpolymorphic antigens has yet to be unraveled.[9]

In addition, blood group genotyping provides a means to identify the (absence of) antigen expression when test antisera from immunized donors are rare or notoriously unreliable, the latter for example being the case for the Do^b antigen in the Dombrock blood group system.[57] As such, genotyping of identified SNPs can function as an additive to conventional serological typing,[53,57] especially since a wide variety of low- and high throughput platforms are now available.[53] As an example, the European Bloodgen consortium has developed a Luminex beads array capable to genotype over 116 blood group-specific SNPs (BLOODchip). The last CE-marked version of this array types 29 SNPs that together determine 37 antigens among 10 blood group systems (RhCE, Kell, Kidd, Duffy, MNS, Diego, Dumbrock, Colton, Cartwright, and Lutheran) within only four hours on the basis of a sample.[58] Specifically paid attention for during the developmental process, it has gained a high sensitivity to predict unusual Rh variants, although ABO and RhD typing is currently not reliably accurate for diagnostic clinical practice.[59]

Yet, simply replacing conventional serological typing by DNA-based methods is currently precluded. First, for antigens that are not the direct product of an allele, the phenotype may not be easily predicted by the genotype. In this regard, DNA-based methods can have problems in discriminating the O allele from an A^1 allele, because inactivating mutations in the glycosyltransferase gene may occur at many different places in the coding region of the gene.[9] Second, exchange of DNA sequences between closely linked genes may induce all kinds of rare variant gene products.[53] Third, the high costs of these genotyping techniques currently do not justify a general introduction.[9] Finally, false prediction of a positive antigen status can occur if an inactivating mutation affecting antigen expression (i.e. null phenotype) is not included in the assay.[53]

Despite the above, molecular typing has deserved its credits over the past decades and will become more and more important. As such, current useful applications of DNA-based typing in transfusion medicine involve fetal Rh DNA typing, red cell antigen typing for the already alloimmunized recipient, determining antigenic phenotypes in patients for whom this is serologically impossible (i.e. recently transfused, autoimmune antibodies), and donor screening aiming to detect rare blood group phenotypes.[9,60]

In future, serological typing especially for post-transcriptional determined blood groups like ABO will remain indispensable, however, genotyping likely will become more widely available for red cell recipients expected to easily develop alloantibodies. In addition, mass-scale genotyping of blood donors will support the expansion of the antigen-negative red cell units inventories. Consequently, a more universal application of molecular technologies for both donors and recipients of red cells will undoubtedly

become integrated into the clinical practice. As such, these new technologies add to the prevention of alloimmunization.

Red blood cell alloimmunization: prevention

Red cell antigen matching: current practices

Currently, all recipients of donor red cell units in developed countries receive ABO and RhD compatible blood, hereby avoiding direct ABO incompatibility-mediated hemolysis and exposure to the highly potent antigen D.[61,62] Despite the effectiveness of antigen matching at reducing alloimmunization rates,[63-66] alloantibodies against other antigens are not prevented by these general measures and attribute to morbidity and even several deaths yearly.[44,45]

Although a complete antigenic donor-recipient phenotype match would theoretically eliminate all elective transfusion-induced alloimmunizations, this practice is extremely expensive and labor consuming. The next best alternative would be to select donor units matched at least on the most immunogenic antigens for at least the patients with a higher than average alloimmunization risk. In line with this, patients with myelodysplastic syndrome, and auto- and/or alloimmunized patients in the Netherlands receive C, c, E, e, and K matched blood.[52] Similarly, women under 45 years of age receive c, E, and K compatible units as alloimmunization might severely complicate future pregnancies if the fetus expresses these antigens by paternal inheritance. Patients with hemoglobinopathy in addition receive Fy^a, and preferentially Jk^b and Ss matched blood.[52] Although several studies have clearly demonstrated patients with sickle cell disease to benefit from extended matching,[63,66,67] for other patient populations, such as women of childbearing age and patients with myelodysplastic syndrome, current practices have been merely based on expert opinions.

Determinants of red cell alloimmunization

Earlier reports, including one of our own, illustrated that only a minority of the intensively transfused patient population eventually develops alloantibodies despite repeated exposure to hundreds of different non-self red cell antigens.[68,69] Whether or not a red cell recipient ultimately mounts an alloimmune response thus is not a default occurrence, but instead seems to depend on various, currently ill defined, factors related to exposure loads, the antigen, and the recipient's immune system's condition.

Exposure

A first and absolute prerequisite for transfusion-induced alloimmunization is exposure to a non-self red cell antigen. In this regard, allogeneic red cell exposure [68-70] and, more specifically, exposure to high immunogenic alloantigens, are important determinants of

alloimmunization that increase the chance of alloimmunization. Additionally and as specified in **chapter 2**, this chance further depends on the likelihood to encounter a non-self antigen and thus on the antigen distribution among both the recipient and the donor population. Consequently, ethnicity determined blood group variations between e.g. patients with thalassemia or sickle cell disease and their donors, the latter in the Netherlands in general being from Caucasian background, at least partly explains why these patients have a larger alloimmunization risk.[6,54,70,71]

Antigen immunogenicity

Second, the potency of an alloantigen to trigger an adaptive immune response is of importance and is defined as the antigen's immunogenicity. Here, the probability that non-self peptide fragments fit into the pocket of a human leucocyte antigen (HLA) class II and are subsequently presented to $CD4^+$ T cells, logically increases with the number of non-self epitopes on the polypeptic structure of the antigen. Similarly, the likelihood that multiple antigen-specific naive B cells are present will increase with the degree of foreignness of the antigen. Even though a substantial homology between the *RhD* and *RhCE* genes exists,[6] the complete absence of the D protein in RhD-negative individuals guarantees exposure to several non-self epitopes when RhD positive red cells are transfused. [72,73] The D antigen in this regard represents the most immunogenic red cell antigen[74] with anti-D formation observed in around 30% of the transfused patient population and in up to 80% of healthy volunteers after one single transfusion.[61,62,75] Consequently, as polymorphic red cell antigens within minor blood group systems differ to a far lesser extent from one another, this might be one reason why they are far less immunogenic than D.

Another concept of red cell antigen immunogenicity involves the non-exofacial polymorphic structures (NEP) hypothesis, proposed by Zimring et al.[76] B cells, via their BCR, only recognize molecular structures presented on the outer membrane of red cells. However, by internalizing (parts of) the red cell and subsequently presenting both extracellular (B cell epitope) and NEP structures, T cells specific for epitopes other than the (extracellular) B cell epitope might be able to stimulate these B cells. Thus, next to the number of B cell epitopes, the number of NEPs will also determine the immunogenicity of the antigen.[76] Similarly, the NEP mechanism may induce activation of autoreactive B cells even when autoantigen specific T cells are absent.

Patient specific characteristics

Third, the patient's genetic constitution (nature) as well as nurture-related characteristics e.g. environmental factors and the disease related factors, will likely govern the immune system's ability to evoke a red cell alloimmune response. Available evidence supports the view of a 'responder population', i.e. patients responding to red cell alloantigens at much higher rates than the general transfused population.[68]

Several studies implicated polymorphisms in the human leucocyte antigen (HLA) genes to affect alloimmunization. Even when a recipient is exposed to antigenic incompatible donor red cells, an alloimmune response will only be initiated when these incompatible antigens subsequently are presented to cells of the adaptive immune system. In this regard, the likelihood of a naive $CD4^+$ T cell to encounter a foreign red cell peptide in the context of HLA class II both depends on the foreignness of the antigen as well as on the HLA type itself. Thus, the patient-specific HLA type could be responsible for shaping the T cell repertoire and thereby determining the likelihood of antigen-specific B cell activation.[72] Indeed, the high immunogenicity of K might mirror the low HLA restriction of this antigen,[77,78] while for Kidd and Duffy antigens only particular HLA types seem to predispose to antibody induction.[78-81] Next to HLA, mutations in genes of importance to the functioning of both the innate and adaptive immune system might influence alloimmunization as well (e.g. cytokine, chemokine, surface receptors, and intracellular signaling pathway genes). These have not been broadly investigated so far. One small study in sickle cell disease patients reported on a potential role for the *TRIM21* gene, which is important for intracellular antibody neutralization of coated virions and stimulation of several pro-inflammatory transcription pathways.[82,83] However, these results might have been due to chance as two other studies were not able to confirm this.[84,85] In a subsequent case-control genome-wide association study, a suggestive association between SNPs in the inhibitory *Toll-like receptor-10* gene and red cell alloimmunization was reported, albeit again the small sample size of the study and the lack of significance prevent firm conclusions.[84] In conclusion, except from some suggestions made for an existing association between alloimmunization and HLA type, current available knowledge on genetic variations is insufficient to identify the high-risk patient population.

With regard to clinical conditions, some more evidence is available. Many studies have highlighted the high alloimmunization prevalences among patients with sickle cell disease and thalassemia. In addition to the large antigenic disparity between these patients and their mainly Caucasian red cell donors as well as their often continuous dependency on red cell transfusions,[54,71,86,87] a potential influence of disease-related chronic inflammation has been suggested to contribute to high alloimmunization risks.[88-90] In line, several murine studies have consistently marked experimentally induced inflammation to be a major determinant of alloimmunization.[30,36,37,91]

Finally, an enhanced alloimmunization susceptibility has been reported for patients with myelodysplastic syndrome (MDS).[92-94] Yet, these prevalence-deduced results should at least be ascribed to the patients' high transfusion burden.[94] A possible attributable influence of other disease related features, e.g. intrinsic biological disease characteristics and treatment modalities, has so far been unclear, as various conclusions have been proposed.[92,93,95,96] Evidence for risks in patients with other oncological disease entities has been lacking, except for one study reporting comparable risks for oncologic and non-oncologic patients. However, this study based its conclusions on a patient population

with a heterogeneity of oncological diagnoses.[97] Since the degree of treatment-induced immunosuppression will be closely related to the specific oncologic diagnosis, alloimmunization rates observed in a mixed oncologic patient population might not correlate well to disease-specific risks.

The R-FACT study: Risk Factors for Alloimmunization after red blood Cell Transfusions

Taken together, there is an urgent need to advance our understanding of the process of alloimmunization. A thorough identification of conditions critical for red cell alloimmunization would help to better discriminate patients likely to induce alloantibody formation from those not responding. As such, this knowledge could support tailoring matching strategies, hereby aiming to eradicate transfusion-induced alloimmunization and its clinical consequences.

The establishment and implementation of a so-called 'alloimmunization prediction score' in this respect might serve as an important tool for this goal. Such a validated score might enable the physician to allocate extended matched blood principally to the high-risk patient who will benefit most from extended matched blood. Consequently, this could initiate the alignment and optimization of donor management, with sizes and variations of blood inventories being adjusted to specific patient needs.

With this perspective in mind, the R-FACT study was initiated in 2008. Its case-control study design enables to efficiently investigate the associations of several determinants with a rather low-prevalent outcome (i.e. alloimmunization). By using an incident new-user cohort as source population and subsequently matching non-alloimmunized controls to alloimmunized cases based on the number of (lifetime) transfusions, selection of existing cases as well as of prevalent transfused recipients was avoided. This 'incidence-density sampling strategy' guarantees matched controls to form a representative sample of the non-alloimmunized transfused source population and to have been exposed to at least the same number of transfusions as their matched cases.[98,99] Yet, as controls did not develop alloantibodies despite their cumulative exposure being at least equivalent to that of cases, identification of other risk-modifying factors is permitted.

By using this R-FACT study design first in a two-center source population of 5,812 patients, including 156 cases and 312 randomly selected controls, our group previously concluded and reported that the storage time of red cell units, evaluated for a clinically relevant range between 7 and 28 days, is not associated with the post-transfusion risk of alloimmunization.[100] In addition, it was illustrated that only the total number of red cell units received rather than the time frame over which these units are received (i.e. massive versus dispersed) determines red cell alloantibody formation.[69,101]

Outline of this thesis

Since the initiation of the R-FACT study and its first published reports, the two-center R-FACT patient cohort has been expanded to a cohort of 24,063 newly-transfused patients who were consecutively transfused in six different hospitals in The Netherlands. Participating hospitals include three academic hospitals (Leiden University Medical Center, Leiden; University Medical Center Utrecht, Utrecht; and VU Medical Center, Amsterdam) and three non-academic hospitals (Catharina Hospital, Eindhoven; Jeroen Bosch Hospital, 's Hertogenbosch; and HagaHospital, The Hague). Enlarging our case-control cohort as such allows identification of additional conditions that impact the red cell alloimmunization process, either related to common disorders or to more specific, rare disease entities. The above mentioned studies by Zalpuri et al primarily focused on the association between donor-related factors and red cell alloimmunization.[69,100] Continuing this research line, but now focusing on recipient-related factors, the studies presented in this thesis specifically set out to identify clinical conditions determining the process of red cell alloimmunization.

As one of the most important elements of red cell matching strategies, **chapter 2** provides qualitative and quantitative data on the intrinsic potency of several red cell antigens to induce red cell alloimmunization. If one fulfills the criteria to receive extended matched blood, the likelihood of allogeneic antigen exposure as well as the antigens' immunogenicities will need to be weighed against one another in order to decide on the optimal antigen subset this patient deserves to be matched for. In **chapters 3-6**, we subsequently examine which of several potential risk-modifying clinical conditions need to be taken into account. We here consecutively study the influence of various types of infections with their associated degrees of inflammation (**chapter 3**), the critical role of the spleen in red cell alloimmunization (**chapter 4**), the effect of general immunosuppressive therapeutic agents (**chapter 5**), and the association of various hematological malignancies and solid cancers with red cell alloimmunization (**chapter 6**). Regarding the latter, disease associated treatment regimens as potential strong immunomodulating factors were specifically assessed and found to be of major influence. **Chapter 7** highlights and discusses several of the topics of this thesis and postulates perspectives for future research within the field.

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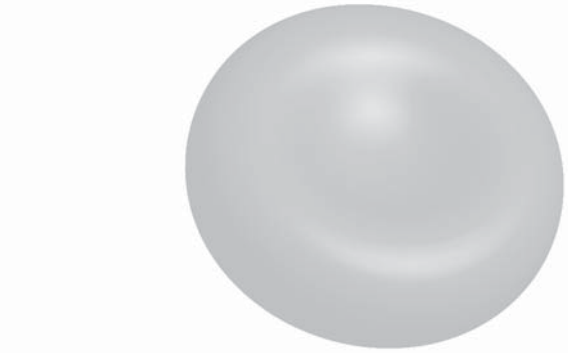

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RED CELL ALLOIMMUNIZATON IN RELATION TO ANTIGENS' EXPOSURE AND THEIR IMMUNOGENICITY: A COHORT STUDY

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Abstract

Background

Matching donor red cells on recipient antigens prevents alloimmunization. Knowledge about the immunogenicity of red cell antigens can help optimize risk-adapted matching strategies. We set out to assess the immunogenicity of red cell antigens.

Methods

In an incident new-user cohort of 21,512 previously non-transfused, non-alloimmunized Caucasian patients receiving non-extended matched red cell transfusions in six Dutch hospitals between 2006 and 2013, we determined the cumulative number of mismatched red cell units per patient. Missing antigen data were addressed using multiple imputation. Using Kaplan-Meier analysis, we estimated cumulative alloimmunization incidences per mismatched antigen dose as a measure of immunogenicity.

Findings

Alloantibodies occurred in 2.2% (474/21,512) of all transfused patients with cumulative alloimmunization incidences increasing up to 7.7% (95% confidence (CI) interval 4.9-11.2) after 40 units received. The antigens C, c, E, K, and Jk^a were responsible for 78% of alloimmunizations in our cohort. K, E, and C^w were the most immunogenic antigens (cumulative immunization incidences 2.3% (CI 1.0-4.8), 1.5% (CI 0.6-3.0), and 1.2% (CI 0.0-10.8) after 2 mismatched units). These antigens were 8.7, 5.4, and 4.6 times as immunogenic as Fy^a. This immunogenicity order was followed by e, Jk^a, and c (1.9, 1.9, and 1.6 as strong as Fy^a).

Interpretation

Red cell antigens vary in their potency to evoke a humoral immune response. Our findings highlight that donor-recipient red cell matching strategies will be most efficient when primarily focusing on prevention of C, c, E, K, and Jk^a alloimmunization. Matching for Fy^a is of lower clinical relevance. Ethnicity determined variations of antigen frequencies prevent extrapolating these conclusions to non-Caucasian populations.

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Introduction

Exposure to foreign red cell antigens may induce alloimmunization. Notwithstanding current ABO/RhD matching and stringent antibody screening policies, life-threatening hemolytic reactions resulting from boosting of previously induced alloantibodies still complicate red cell transfusions.[5,6] Moreover, previous alloimmunizations demand extensive laboratory efforts and can result in delays in finding compatible donor blood.

A complete antigenic donor-recipient phenotype match would theoretically eliminate all transfusion induced alloimmunizations, however, meets countless logistical and financial challenges. The next best alternative is to select donor units matched at least on the most immunogenic antigens for patients at high risk. In line with this, patients with myelodysplastic syndrome, and auto- and/or alloimmunized patients in the Netherlands are advised to receive CcEe and K matched blood, while patients with hemoglobinopathy additionally receive Fy^a and preferentially Jk^b and Ss matched blood as well.[7] As alloimmunization can severely complicate subsequent pregnancies, women under 45 years of age receive cE and K matched blood.[7] These matching strategies are based on broad expert consensus on the antigens' immunogenicity i.e. their intrinsic potency to stimulate humoral immune responses. RhD is without doubt the most immunogenic antigen and is followed by K.[1] However, data on the relative immunogenicity of several other antigens is conflicting,[1-3] requiring additional observational evidence.

We set out to quantify antigen-specific alloimmunization rates in relation to the cumulative number of mismatched transfusions per patient as a measure of the intrinsic immunogenicity of red cell antigens. This knowledge will enable an evidence-based design of optimizing matching strategies, balancing benefits against costs and logistic aspects.

Methods

Study design and setting

We performed an incident new-user cohort study among patients consecutively transfused in three university hospitals and three non-university hospitals in the Netherlands. We included all previously non-transfused and non-alloimmunized patients who received at least one red cell transfusion during the study period, provided the availability of at least one pre- and post-transfusion antibody screen. 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).

We used the following safeguards. First, patients alloimmunized within seven days of a mismatched transfusion were excluded as they more likely presented boosting rather than primary alloimmunization. Second, the records of alloimmunized patients were consulted against the nationwide Dutch alloimmunization registry (TRIX)[8] for earlier alloantibody detection in other hospitals. Third, allo- rather than auto-immunization was verified based on the patient's phenotype. Fourth, as CcEe and K phenotypes for over 99% of donor blood units were available, we excluded CcEe and/or K alloimmunized patients with no identifiable mismatched transfusion. Finally, we set out to exclude all patients who received more than only routinely (ABO/RhD) matched units. To that aim, we excluded women below 45 years of age who, in line with Dutch guidelines,[7] receive c, E, and K compatible units. Auto-immunized patients without alloimmunization, and hemoglobinopathy patients were excluded as they usually receive extended matched units as well.[7] In addition, ethnicity determined differences in antigen distribution between hemoglobinopathy patients and the Dutch, generally Caucasian, donor population would have led to unrepresentative alloimmunizations, further compromising the validity of antigen immunogenicity estimates. We did not exclude patients with myelodysplastic syndrome since, despite Dutch guideline advises,[7] they do not receive extended matched blood (unpublished personal data). Infants under 6 months of age were excluded as poor antibody responses during the first months of life are reported.[9]

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

Detection of 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 screening cells 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 minimally heterozygously. The presence of C^w, Lu^a, Wr^a, and Kp^a is not mandatory on commercially available screening cells.[7]

Screening was performed using a 3-cell panel including an indirect antiglobulin test (LISS Diamed ID system (Bio-Rad, Cressier, Switzerland), or Ortho Biovue ID system (Ortho Clinical Diagnostics, Raritan NJ, United States)) and subsequent antibody identification. The antigen C^w was present on 93% of all 3-cell panels used during the time frame of this study.

Data acquisition

Routinely stored data on red cell transfusion dates, product unique identification number, dates and results of antibody screens, antibody specificity, and patient's date of birth and sex were gathered from the hospitals' electronic laboratory information systems.

Available antigen phenotypes for all transfused products were delivered by Sanquin Blood Supply, Amsterdam, The Netherlands.

Statistical analyses

Overall and antigen-specific cumulative incidence of alloimmunization

All included patients were followed up and labelled as 'alloimmunized' upon a first-time alloantibody identification or as 'non-alloimmunized' if the alloantibody screen remained negative.

Cumulative numbers of transfused red cell units corresponded to the total number of units received up to the last available negative screen for non-alloimmunized patients and up to the last verified or presumed antigen-mismatched unit that preceded the first positive screen for alloimmunized patients (Figure S1). Consequently, transfusions received after these screens were not taken into account. Using Kaplan-Meier survival tables, we then calculated overall cumulative alloimmunization incidences and subsequently cumulative incidences per antigen.

Cumulative incidences with 95% confidence intervals (CI) were calculated with Graphpad Prism version 6, using the exponential Greenwood formula. The association between sex and alloimmunization incidences was assessed with the log-rank test using SPSS version 20.0. P-values <0.05 were considered to be statistically significant.

Immunogenicity of red cell antigens

To evaluate the immunogenicity of various red cell antigens, we calculated cumulative alloimmunization incidences per antigen according to the total number of mismatched (i.e. antigen-positive) units a patient had received (Figure S1). For this purpose, only those at risk for alloimmunization against a given antigen should be considered, i.e. patients lacking expression of this antigen. As antigen phenotyping of non-alloimmunized patients is limited to ABO and RhD in the Netherlands, we established 'antigen-negative cohorts' comprising all (per definition antigen-negative) patients alloimmunized to a given antigen plus a randomly sampled subgroup of non-alloimmunized patients. Although patients in these sampled subgroups not necessarily all lacked expression of the corresponding antigen, they only functioned as a representative for the true antigen-negative, non-alloimmunized individuals in the source population. We based the sampling sizes of these subgroups on known antigen frequencies in the Caucasian population.[10] Thus, as 29% of the Caucasian population express the E antigen,[10] 71% of our source population lack E expression. Given an extensive size of the source population, the numbers of E-positive units received by the randomly sampled 71% will closely correspond to the numbers received by the true E-negative, non-alloimmunized individuals in the source population. This sampling method is not likely liable to selection bias as expression of a given antigen is not associated with the amount of alloantigen exposure in non-extended matched individuals.

To address missing donor phenotypes, we used multiple imputation to complete the dataset. Details on frequencies of missing data and the used method are presented in table S1.

We then calculated cumulative alloimmunization incidences for each antigen according to the total number of mismatched units, except for antigens with over 50% missing data.

Finally, we compared antigen-specific cumulative alloimmunization incidences with those of Fy^a. Previously, comparisons with K were made.[1-4] However, the rate of censoring between baseline and *N* transfusions may compromise the validity of estimated cumulative incidences (for illustration, see Supplementary Box 1). Consequently following the antigen's low frequency, the reliability of estimated cumulative anti-K incidences decreases with the number of K-positive units exposed as only a few patients are repeatedly exposed to K. As in former reports Fy^a immunogenicity was in the middle of the extremes[1-4] and as (except for S) the probability that a random individual both does not express a given antigen and is exposed to this antigen is the highest for Fy^a,[10] we chose Fy^a as the reference. Due to the above mentioned issues regarding censoring, we only calculated cumulative alloimmunization incidences per antigen for antigen-negative cohorts containing at least 200 non-censored patients.

Role of the Funding Source

This study was not externally funded. The corresponding author had full access to all of the data and the final responsibility to submit for publication.

Results

A total of 54,347 patients received their first red cell transfusion during the study period, of which 21,512 patients fulfilled the inclusion criteria. Figure 1 presents numbers of patients per exclusion criterion. The majority of the 32,835 patients deemed ineligible were excluded while no antibody screen was performed after their single transfusion episode (N=25,037).

Table 1 presents patient demographics. Patients received a median of 4 (interquartile range (IQR) 2-8) units of red cells during a median follow-up period of 86 (IQR 14-395) days. In 474/21,512 patients (2.2%), 536 first formed alloantibodies were detected, the majority being against C, c, E and K antigens (table 2). In 51 patients, the alloantibody was detected within the second week after the first documented antigen-mismatched transfusion.

Figure 1 Study flow diagram.

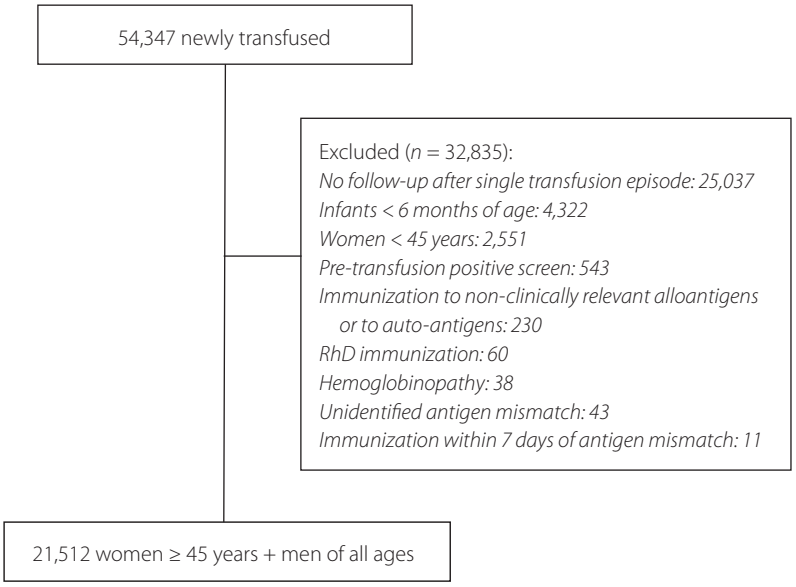


Table 1 Patient demographics of the study population.

	N=21,528
Men (N, %)	12,511 (58.1)
Age in years at 1st transfusion, median (range / IQR)	67.7 (0.5-107.2 / 57.5-76.9)
Cumulative units	153,429
Units per patient, median (range / IQR)	4 (1-462 / 2-8)
Follow-up in days, median (range / IQR)	86 (1-3155 / 14-395)
Alloimmunized patients	474
Alloimmunization frequency (%)	2.2

Follow-up period = period in days from 1st red cell transfusion up to the last negative antibody screen for non-alloimmunized patients, and up to the first positive alloantibody screen for alloimmunized patients.
IQR = interquartile range.

Table 2 Specificity and frequency of first-time formed clinically significant alloantibodies (N, %).

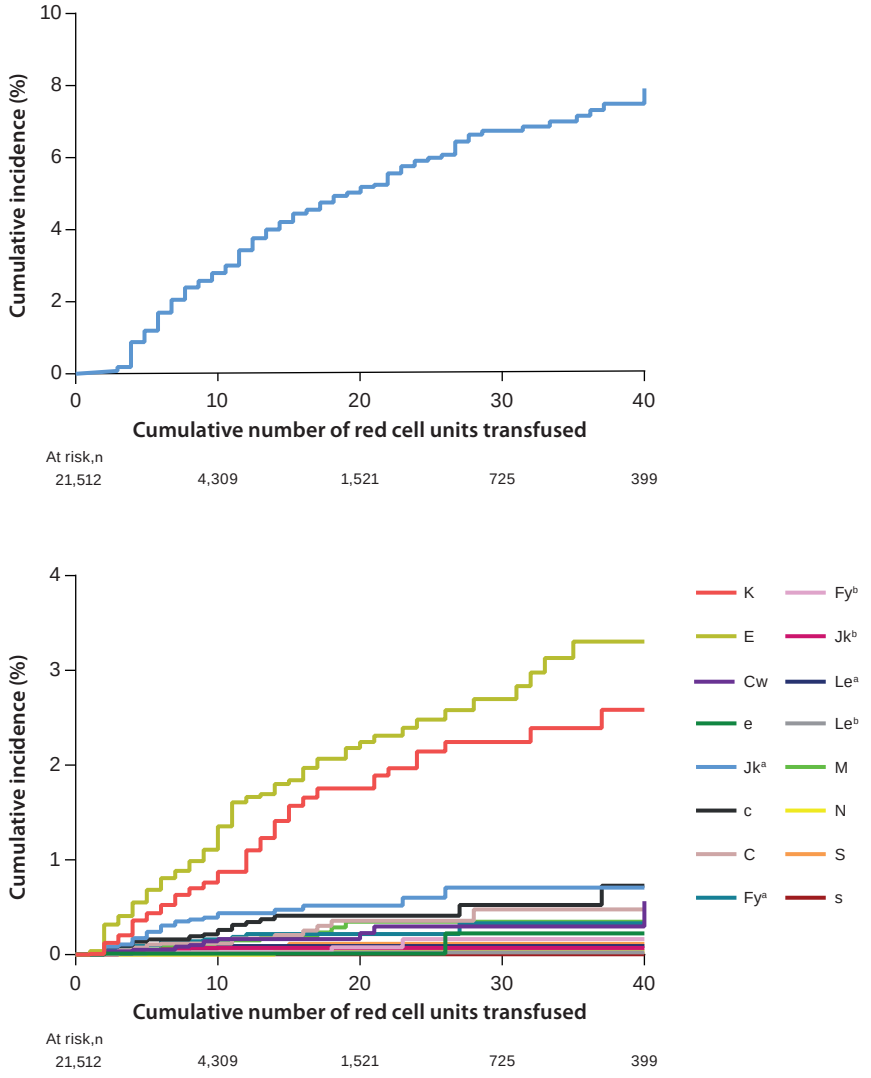
Alloantibody specificity	All N=21,512	RhD pos N=18,191 (84.6%)	RhD neg N=3,321 (15.4%)
anti-C	22 (0.10)	18 (0.10)	4 (0.12)
anti-c	37 (0.17)	37 (0.20)	0 (0)
anti-E	177 (0.82)	173 (0.95)	4 (0.12)
anti-e	4 (0.02)	4 (0.02)	0 (0)
anti-K	122 (0.57)	95 (0.52)	27 (0.81)
anti-C^w	19 (0.09)	18 (0.09)	1 (0.03)
anti-Fy^a	24 (0.11)	21 (0.12)	3 (0.09)
anti-Fy^b	5 (0.02)	4 (0.02)	1 (0.03)
anti-Jk^a	50 (0.23)	41 (0.23)	9 (0.27)
anti-Jk^b	7 (0.03)	7 (0.04)	0 (0)
anti-Lu^a	31 (0.14)	29 (0.16)	2 (0.06)
anti-Lu^b	0 (0.0)	0 (0)	0 (0)
anti-Le^a	8 (0.04)	6 (0.03)	2 (0.06)
anti-Le^b	3 (0.01)	3 (0.02)	0 (0)
anti-M	18 (0.08)	14 (0.08)	4 (0.12)
anti-N	1 (0.01)	1 (0.01)	0 (0)
anti-S	8 (0.04)	7 (0.04)	1 (0.03)
anti-s	0 (0)	0 (0)	0 (0)
All antibodies	536	478	58
Number of cases	474 (2.20)	419 (2.30)	55 (1.66)

Overall cumulative incidence of alloimmunization

The overall cumulative alloimmunization incidence increased to 7.7% (CI 4.9-11.2) after 40 red cell units transfused (figure 2 upper panel). Antibodies to E and K were formed at the highest rates (figure 2 bottom panel). Table 3 presents cumulative alloimmunization incidences against various sets of antigens referenced to the overall cumulative alloimmunization incidence after 40 units transfused. Hence, in this ABO/RhD matched patient cohort, 78.6% of alloimmunizations were due to immunizations against C, c, E, K, or Jk^a.

Frequencies of anti-c and anti-E mirrored *RHD* and *RHCE* gene linkage. In this respect, anti-c was only formed by RhD-positive patients and absence of RhD expression led to significantly less E alloimmunizations with cumulative allo-E incidences of 1.7% (CI 0.0-32.0) and 3.7% (CI 1.4-7.9) after 40 red cell units received for RhD-negative and RhD-positive patients, respectively (log-rank $p<0.0001$). RhD phenotype did not modulate the risk of immunization against other, non gene-linked, red cell antigens (figure S2).

Figure 2 Cumulative alloimmunization incidences in the general population (upper panel) and according to antigen (lower panel).



Cumulative alloimmunization incidences as a function of cumulative red cell units exposed. Antibodies to E and secondary to K were formed at the highest incidence rates.

Table 3 Cumulative alloimmunization incidences (%) against various sets of antigens referenced to the overall cumulative alloimmunization incidence.

	Cumulative alloimmunization incidence (%)*	Proportion of all antibodies (%)
All antibodies	7.66 (4.89-11.24)	100
E	3.32 (1.20-7.25)	43.3
cE	3.59 (1.40-7.47)	46.9
cEK	5.20 (2.71-8.87)	67.9
cEeK	5.39 (2.84-9.12)	70.4
CcEK	5.74 (3.15-9.42)	74.9
cEK+Jk^a	5.67 (3.15-9.23)	74.0
CcEK+Jk^a	6.02 (3.46-9.55)	78.6
CcEK+Jk^a+Fy^a	6.24 (3.66-9.76)	81.5
CcEK+Jk^a+Fy^a+C^w	6.39 (3.81-9.88)	83.4

***** Cumulative alloimmunization incidences as calculated for 40 red cell units transfused.

Among patients over 45 years of age, women showed higher Rh (i.e. CcEe and C^w) and K alloimmunization incidences compared to men (7.9% (CI 3.2-15.3) versus 5.2% (CI 2.0-10.9) after 40 units received, log-rank p<0.0001, figure S3). Alloimmunization to non-Rh/non-K antigens did not differ between male patients under and above 45 years of age (log-rank p=0.705).

Immunogenicity of red cell antigens

The antigen's specific immunogenicity was derived from cumulative alloimmunization incidences according to cumulative antigen mismatched units received. Substantial missing antigen data for Le^b, Lu^a, and Lu^b prevented immunogenicity calculations for these antigens (table S1).

Cumulative alloimmunization incidences after exposure to only two antigen-positive units were 2.3% (CI 1.0-4.8), 1.5% (CI 0.6-3.0), and 1.2% (CI 0.0-10.8) for K, E, and C^w respectively. Less extensive responses were observed for e, Jk^a, and c (figure 3 upper panel, table 4). Following a similar amount of E exposure, anti-E formation did not differ between RhD-negative and RhD-positive patients (log-rank p 0.44).

The calculated relative immunogenicity of K, E, and C^w was 8.7, 5.4, and 4.6 times higher than Fy^a after only two antigen-positive units. For e, Jk^a, and c these rates were 1.9, 1.9, and 1.6, respectively. Relative immunogenicity rates were lower for the other antigens (figure 3 bottom panel).

Table 4 Adjusted cumulative red cell alloimmunization incidences according to numbers of antigen-mismatched units received in the antigen-negative cohorts.

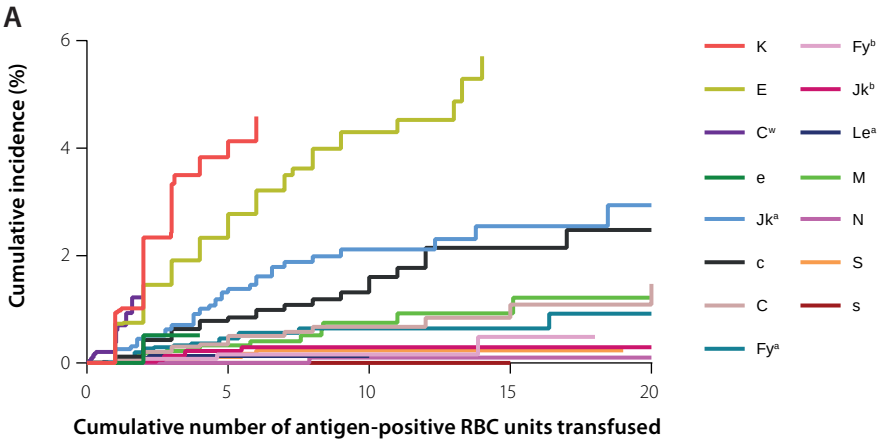
	Adjusted cumulative alloimmunization incidence, % (CI)														
Mismatched units (N)	C	c	E	e	K	C^w	Fy^a	Fy^b	Jk^a	Jk^b	Le^a	M	N	S	s
1	0.05 (0.00-8.78)	0.12 (0.00-6.10)	0.73 (0.21-2.00)	0.00 (0.00-0.00)	0.94 (0.27-2.53)	0.38 (0.00-5.42)	0.09 (0.00-4.33)	0.00 (NC)	0.26 (0.00-3.40)	0.00 (NC)	0.06 (0.00-4.74)	0.09 (0.00-7.25)	0.00 (NC)	0.04 (0.00-7.91)	0.00 (NC)
2	0.21 (0.00-3.66)	0.43 (0.01-3.55)	1.46 (0.61-2.99)	0.51 (0.00-26.76)	2.34 (0.95-4.84)	1.23 (0.02-10.78)	0.27 (0.01-2.87)	0.08 (0.00-16.81)	0.51 (0.03-3.24)	0.02 (0.00-30.92)	0.10 (0.00-3.84)	0.18 (0.00-5.02)	0.00 (NC)	0.08 (0.00-5.34)	0.00 (NC)
5	0.50 (0.01-4.20)	0.85 (0.07-4.24)	2.77 (1.34-5.07)		4.13 (1.53-8.80)		0.46 (0.02-3.50)	0.16 (0.00-14.66)	1.38 (0.24-4.79)	0.23 (0.00-6.72)	0.13 (0.00-4.40)	0.33 (0.00-5.14)	0.00 (NC)	0.12 (0.00-6.13)	0.00 (NC)
10	0.67 (0.02-5.11)	1.60 (0.18-6.68)	4.30 (1.96-8.06)				0.64 (0.03-4.36)	0.16 (0.00-14.66)	2.11 (0.46-6.29)	0.30 (0.00-6.88)	0.13 (0.00-4.40)	0.75 (0.01-7.34)	0.10 (0.00-37.40)	0.24 (0.00-7.21)	0.00 (NC)
15	0.84 (0.02-6.81)	2.15 (0.28-8.17)					0.64 (0.03-4.36)	0.49 (0.00-26.04)	2.55 (0.51-7.83)	0.30 (0.00-6.88)		0.94 (0.02-8.28)	0.10 (0.00-37.40)	0.24 (0.00-7.21)	0.00 (NC)
20	1.48 (0.02-12.15)	2.47 (0.29-9.55)					0.92 (0.01-9.23)		2.94 (0.49-9.65)	0.30 (0.00-6.88)		1.22 (0.02-10.47)	0.10 (0.00-37.40)		

Only data from non-censored cohorts of at least 200 subjects are presented. CI = 95% confidence interval. NC = non-computable due to the absence of events.

When only C^w alloimmunized patients with a verified C^w mismatched transfusion were included in the above analysis (N=10, those with assumed C^w mismatched transfusions excluded), C^w alloimmunization incidences did not change substantially (1.2% (CI 0.0-10.8 versus 1.0% (CI 0.0-12.8) after 2 C^w positive units transfused, log-rank p=0.10).

As an additional sensitivity analysis, results of antigen immunogenicity calculations repeated in only men were identical (figure S4).

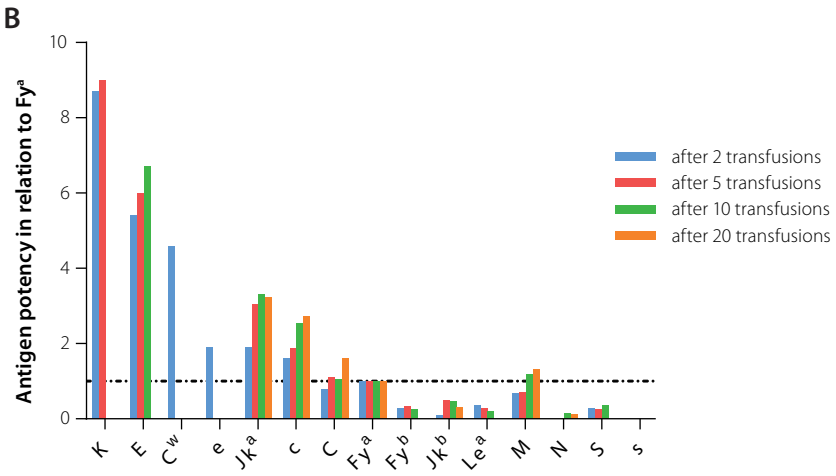
Figure 3 The relative immunogenicity of specific antigens presented by cumulative alloimmunization incidences as a function of antigen exposure.



At risk,n					
K	19,274	324	35	7	2
E	15,122	1,759	506	195	95
C^w	20,648	20	3	1	0
e	433	172	91	52	36
Jk^a	4,890	1,665	718	378	227
c	4,247	1,516	683	372	229
C	6,758	1,923	774	403	259
Fy^a	7,181	2,193	897	445	255
Fy^b	3,583	1,278	560	290	172
Jk^b	5,482	1,766	725	363	209
Le^a	16,427	1,001	222	70	24
M	4,648	1,610	890	372	225
N	5,895	1,798	753	407	234
S	9,479	2,096	746	350	184
s	2,317	893	410	209	141

(**A**) Antigen-specific cumulative alloimmunization incidences according to number of antigen-positive red cell units received by the antigen-negative patient cohorts, and (**B**) as referenced to Fy^a. K, E, C^w, and to a lesser degree e, Jk^a, and c, are the most immunogenic antigens.
Numbers at risk correspond to total number of patients within the corresponding antigen-negative cohort exposed to at least *N* antigen-positive red cell units. Only data from non-censored cohorts of at least 200 subjects are presented.

Figure 3 Continued.



Discussion

In this study covering 21,512 newly transfused patients, we established estimates of dose-specific red cell alloimmunization risks. In agreement with previous reports, K is most potent in stimulating humoral alloimmune responses. E demonstrates the second highest immunogenicity, and the order of antigen immunogenicity is then followed by C^w, e, Jk^a, and c.

The here established alloimmunization rate of 0.9% after one K-positive unit is five times lower than historically assumed.[1] Moreover, as previous studies did not take into account the cumulative mismatched transfusion burden, contradictory conclusions regarding the immunogenic potency of other red cell antigens, including c and E, have been reported.[1-4] Indeed, the seemingly flattening of the antigens' alloimmunization risk curves illustrates that the chance to alloimmunization diminishes with subsequent antigen exposure. We therefore specifically set out to properly estimate the cumulative number of antigen-mismatched units each patient had received. Sampling specific-sized 'antigen-negative cohorts' for each antigen enabled us to estimate the cumulative antigen exposure within the true antigen-negative individuals. Selection bias did not interfere with this sampling method as the prevalence of a given antigen is not associated with the likelihood of exposure in non-alloimmunized patients.

The validity of our assessment is confirmed by the constant immunogenicity, though diverse alloimmunization rates against c and E, between RhD-negative and RhD-positive patients, as was expected based on the known *RHD* and *RHCE* gene linkage. Transfusing RhD compatible blood has long since been routine practice as this high immunogenic

antigen induces allo-D in around 30% of D-negative transfused patients and in up to 80% of healthy volunteers.[11,12] Due to this gene linkage, RhD matching not only prevents anti-D formation, but in addition effectively protects against E alloimmunization. That is, as only 6% of the Caucasian RhD-negative population express the E antigen,[10] RhD-negative patients are rarely exposed to E. Conversely, as 67% of RhD-positive individuals lack E,[10] 23% of RhD-positive patients risk E alloimmunization with routine RhD matching. Moreover, the comparable anti-E formation after non-self E exposure between RhD-negative and RhD-positive patients mirrors the fact that RhD phenotype does not influence E-immunogenicity, rather that alloimmunization rates reflect (linkage dependent) exposure. Similarly, anti-c was only identified in the RhD-positive patient cohort as a RhD-negative phenotype is approximately always accompanied by expression of c. However, the chance of a RhD-positive patient to be exposed to c being a non-self antigen is 17%.[10]

Current Dutch matching regimens might seem strict compared to those of other Western countries. Despite, for prior auto- and/or alloimmunized patients, as well as for multi-transfused patients, better targeted matching will likely further reduce the risk of (additional) alloimmunization. As our data seem broadly applicable to populations of Caucasian origin, they enable further optimization as well as unification of current nationwide evidence-based guidelines. In this regard, matching seems most profitable for those antigens with relatively strong immunogenicity, moderate frequency, and potential clinical consequences. Hence, red cell transfusions limited to donor units compatible with the high to moderate immunogenic antigens C, c, E, K, and Jk^a would have reduced alloimmunization incidences by 78%. In line with this, and as anti-Jk^a is notorious for causing delayed hemolytic transfusion reactions,[6,13] additional Jk^a matching for the mentioned risk groups seems advisable. Due to its frequency in the Caucasian population and intermediate immunogenicity, matching for Fy^a should be considered optional. The need to additionally match for C^w seems debatable as, although this antigen is highly immunogenic, severe hemolysis by anti-C^w is rare[14] and the chance of subsequent exposure after primary immunization small (2%).[10]

In agreement with recent data from a 15% overlapping patient cohort,[15] we found higher cumulative alloimmunization incidences in women compared to men over 45 years of age, attributed to higher Rh and K alloimmunization rates. This finding is not easily accounted for by boosting of pregnancy-induced alloantibodies as we used several safeguards to exclude previously alloimmunized women. Moreover, despite (pregnancy-induced) alloantibodies commonly disappearing,[16,17] boosting seems an insufficient explanation as non-RhD alloantibodies form in only 0.33% of first trimester pregnancies[18] amounting for 30 of our 238 (12.6%) alloimmunized post-fertile women. Indeed, others have suggested estrogen or even persisting feto-maternal chimerism to modulate alloimmune responses in women.[15,19]

Several factors and possible limitations of our results require discussion.

First, the time needed for an antibody to develop to serologically detectable levels potentially differs per antigen, but these 'lag periods' are currently unknown. Additionally, titers of previously formed alloantibodies can decrease over time to a degree that prior alloimmunizations are no longer detectable by serological tests. Subsequent exposure to the antigen might boost these 'evanescent' alloantibodies. Indeed, 25 to 40 percent of formed alloantibodies will become undetectable over time, with the highest rates reported for anti-Jk^a and anti-C^w.[16,17] However, as evanescence rates largely depend on the time since exposure (for illustration, see Supplementary Box 2), we were not able to estimate the underestimating effect of antibody evanescence on our immunogenicity calculations.

Second, anti-E, anti-C^w, anti-Le^a, anti-Le^b, anti-Lu^a, and anti-M can also occur 'naturally', i.e. secondary to environmental antigen exposure.[10] One might thus wonder whether the high immunogenicity of C^w should not be explained by high numbers of naturally formed anti-C^w. A sensitivity analysis, including only C^w alloimmunizations which were verified (rather than assumed) to be preceded by C^w mismatched transfusions, confirmed our conclusions. In fact, C^w immunogenicity may well have been underestimated as 7% of used screening cell panels did not present C^w and therefore could not detect anti-C^w. Next, some identified anti-M alloantibodies might have been only of IgM class. Although according to Dutch guidelines a reference laboratory should determine whether identified anti-M antibodies are due to warm-reacting IgG antibodies,[7] this procedure has not been routinely followed in the Netherlands so far.

Third, we set out to exclude all previously transfused and alloimmunized patients. Eleven patients presented with a positive screen within seven days after the first antigen-mismatched transfusion and were excluded as these might have reflected boosting. Nevertheless, while boosting periods can extend seven days and might even differ per antigen, we might not have excluded all previously alloimmunized patients. In this regard, 51 of 474 patients (10.8%) tested positive for alloantibodies within the second week after a first mismatched transfusion, while only a subset of those (N=31) were also tested (negative) during the first week after transfusion. Next, due to some unavailable non-Rh/K donor phenotypes, it remains possible that we assumed a few of those phenotypes to be antigen-positive while in fact the alloimmunized individual was never exposed by transfusion. Finally, we cannot exclude that included patients received some transfusions in other hospitals prior or during the study period. Consequently, some overestimation of the antigens' immunogenicity has to be reckoned.

Fourth, we used multiple imputation addressing missing donor antigen phenotypes while, contrasting nearly complete CcEe and K phenotyping, expression of other minor red cell antigens is less extensively determined among Dutch donors. Several reports have emphasized the superiority of multiple imputation over the traditional missing data techniques.[20-22] Considering data missing at random (MAR), limiting analyses to antigens

with less than 50% missing values, and the substantial sample size we performed this method in, our followed approach will likely have produced unbiased and rather accurate estimates of missing values.[22] Our conservative approach, however, consequently disabled us from presenting any estimations on the immunogenicity of Le^b, Lu^a, and Lu^b. While anti-Lu^a antibodies represented 5.7% (31/537) of all detected antibodies, we cannot exclude Lu^a to be of importance in alloimmunization.

Fifth, hemoglobinopathy patients, often frequently transfused but from non-Caucasian background, were not included in the study. While nearly all donors are of Caucasian origin,[23] around 12% of the Dutch population is of origin other than Caucasian.[24] This discordance may have led to a minor deviation of our estimated mismatched transfusions in the non-alloimmunized patients. Reported antigen immunogenicities may thus be slightly overestimated. In general, due to antigenic, immunological, and genetic differences between ethnicities,[25,26] our results should not be extrapolated to populations of other ethnic backgrounds.

Sixth, although anti-D was detected in 60 of our newly-transfused patient population, we were unable to analyse and confirm the previously reported high immunogenicity of the D antigen[11,12] for several reasons. Due to routine RhD matching, only a very small minority of our RhD-negative patients received D-mismatched units. Next, while the cause of anti-D was often not documented, these antibodies could have been due to either unmatched transfusions or recent anti-D administration.

Seventh, alloantibody responses may differ between various patient cohorts e.g. immunosuppressed versus immune activated patients.[25,27,28] This, however, does not affect any of our conclusions as we only compared the immunogenicity of red cell antigens with one another within the same population. Nevertheless, alloimmunization risks will differ between patient cohorts and the here presented incidences should therefore not be generalized to populations other than general transfused patients. Studies in humans aimed at identifying factors of influence on immunization risks are in progress.[28,29]

Finally, we did not adjust for homo- versus heterozygous donor genotypes as a variable of antigen dose. For most antigens, patients will have received mainly heterozygous donor units. As an example, the observed high immunogenicity of E and K is not distorted by minor cumulative dose differences as the homozygous prevalence rates are only 2.4% and 0.21%, respectively.[30] Moreover, we previously did not find an association between massive versus dispersed transfusions on the risk of alloimmunization.[31]

Though beyond the scope of the present study, a few related and relevant subjects should be mentioned. As an optimal preventive matching strategy demands a comprehensively typed donor cohort, high-throughput genotyping might better facilitate rapid and complete typing in the near future.[32,33] Next, antibody formations needs both sensitization of a B cell as well as priming of a naive $CD4^+$ T cell. Thus, in some of our non-alloimmunized patients, alloreactive B cells to a given blood group antigen might

have been present, yet they lacked specific T cells recognizing a peptide as part of this blood group antigen in the context of human leucocyte antigen (HLA). Finally, knowledge on factors modulating subsequent alloimmunization (such as the type of first-time formed alloantibody possibly determining the rate and type of subsequent alloimmunization) might benefit the already alloimmunized patient.

In conclusion, the risk of red cell alloimmunization is related to both antigen exposure and the antigen's immunogenicity. In this to our knowledge largest Caucasian cohort to date with a defined follow-up reaching an eight year period, we determined the immunogenic order of red cell antigens and quantified dose-based immunization risks with K and E being the most immunogenic antigens, followed by C^w, e, Jk^a, and c. Based on the likelihood of alloantigen exposure, the antigens' immunogenicity, and the potential detrimental consequences of anti-Jk^a boosting, we recommend adding Jk^a matching to current CcEe and K based matching strategies, whenever possible and especially in high-risk patients. Matching for Fy^a can be considered, but, as compared to Jk^a, seems of lesser clinical significance. Due to antigenic frequency differences, these conclusions are not generalizable to patients of non-Caucasian background.

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Supplementary material

Supplementary Box 1 The effect of censoring on estimated cumulative incidences.

We calculated cumulative alloimmunization incidence rates only for antigen-negative cohorts containing at least 200 non-censored patients. This cut-off was chosen as the rate of censoring between baseline and *N* transfusions can compromise the validity of estimated cumulative incidences. In case of a low-frequent antigen (i.e. K), only a few patients will repeatedly be exposed to this antigen and the contribution of random error to the estimated cumulative incidence thus could become unacceptably large. With 200 patients under analysis, one random additional alloimmunization event will increase the cumulative alloimmunization rate by maximally 0.5%, which, in our opinion, justifies comparison of one risk curve to another.

For this same reason, we compared cumulative antigen-specific alloimmunization incidences with alloimmunization incidences of Fy^a. Previously, comparisons with K have been made.[1-4] However, the reliability of estimated anti-K cumulative incidences significantly decreases with the number of K-exposed red cell units as only a minority of patients receive multiple K-positive units due to its low antigen frequency (being 9% in the Dutch donor population).[5] As in former reports Fy^a immunogenicity was in the middle of the extreme[1-4] and as the probability of exposure to a non-self red cell antigen for a random individual is highest (except for S) for Fy^a,[5] we chose Fy^a as the reference.

Finally, we present our data as 'adjusted cumulative risk curves'. As alloimmunized patients are censored at the time of immunization, the size of the population at risk is reduced as a result of alloimmunization. Alloimmunization can thereby lead to an overestimation of the cumulative risk, mainly when a large number of immunization events take place. We therefore calculated 'adjusted' numbers of alloimmunized patients, equalling the number of alloimmunized patients that would have received *N* transfusions had they not been censored at the time of alloimmunization. We used these numbers for Kaplan-Meier analysis.

The examples A-C illustrate that the distribution of events and the rate of censoring can have dramatic effects on final cumulative incidences.

A The effect of event distribution on the cumulative risk.

		Early immunizations		Late immunizations	
Transfused units	**Not censored (N)**	**Immunization events (N)**	**Cumulative risk (%)**	**Immunization events**	**Cumulative risk (%)**
1	10,000	50	0.50	0	0
2	9,000	50	1.05	0	0
3	8,100	50	1.66	0	0
4	7,290	50	2.33	0	0
5	6,560	50	3.08	0	0
6	5,910	0	3.08	50	0.85
7	5,310	0	3.08	50	1.78
8	4,780	0	3.08	50	2.81
9	4,300	0	3.08	50	3.94
10	3,870	0	3.08	50	5.18

Fictitious baseline cohort of 10,000 patients, censoring rate 10% per transfused red cell unit. The same number of events induces a higher cumulative risk when these events occur in a smaller cohort.

B The effect of censoring rates on the cumulative alloimmunization risk.

		Censoring rate 10%		Censoring rate 50%	
Transfused units	**Immunization events**	**Not censored (N)**	**Cumulative risk (%)**	**Not censored (N)**	**Cumulative risk (%)**
1	10	10,000	0.10	10,000	0.10
2	10	9,000	0.21	5,000	0.30
3	10	8,100	0.33	2,500	0.70
4	10	7,290	0.47	1,250	1.49
5	10	6,560	0.62	625	3.07
6	10	5,910	0.79	313	6.17
7	10	5,310	0.97	156	12.18
8	10	4,780	1.18	78	23.44
9	10	4,300	1.41	39	43.07
10	10	3,870	1.67	20	71.54

Fictitious baseline cohort of 10,000 patients with a fixed number of 10 alloimmunization events per transfused red cell unit. Censoring rates of 10% versus 50% per transfused red cell unit. A high censoring rate induces a disproportionally increase of the cumulative risk.

C The effect of censoring of immunized patients at time of alloimmunization.

Transfused units	Immunization events (N)	Not censored cohort (N)	Cumulative risk (%)	Adjusted not censored cohort (N)	Adjusted cumulative risk (%)
1	100	10,000	1.00	10,000	1.00
2	100	9,000	2.10	9,090	2.09
3	100	8,100	3.31	8,274	3.27
4	100	7,290	4.63	7,538	4.56
5	100	6,560	6.09	6,879	5.94
6	100	5,910	7.68	6,293	7.44
7	100	5,310	9.42	5,752	9.05
8	100	4,780	11.31	5,277	10.77
9	100	4,300	13.37	4,848	12.61
10	100	3,870	15.61	4,467	14.57

Fictitious baseline cohort of 10,000 patients, censoring rate 10% per red cell transfusion. Not adjusting for censored alloimmunized patients overestimates the cumulative risk after 10 red cell transfusions by 7.1% ((15.61-14.57) / 14.57) * 100.

Supplementary Box 2 Estimated evanescence rates are dependent on the time following antibody induction.

Reported rates of evanesced antibodies[6,7] so far have been based on prevalences (i.e. the frequency of evanesced alloantibodies at a certain time point) rather than on incidences (i.e. the frequency of evanesced alloantibodies according to the time following exposure). These studies reported evanescence rates as high as 25 to 40% for anti-Jk^a and anti-C^w.

When considering these numbers, one should realize that the frequency of evanesced antibodies increases significantly with increasing time since antibody induction. Alloantibodies against antigens of moderate frequency will in general form rather early during the transfusion history as with every transfusion the likelihood to be exposed to this alloantigen is rather high. In contrast, induction of alloantibodies against low or high frequent antigens will be more evenly distributed along the transfusion history. Thus, even though two types of antibodies evanescence at the same rate, the observed frequency of evanesced antibodies will diverge with the number of red cell units exposed as illustrated in a fictitious example for anti-A and anti-B here below. In conclusion, reported rates of evanesced antibodies are highly dependent on the chance of alloantigen exposure and thus on antigen population frequencies. Addressing evanescence into incidence-based alloimmunization calculations is only possible with data on serological follow-up at multiple fixed times after antigen exposure being available.

time event	anti-A (N)	evanesced anti-A	Cumulative (non-evanesced) anti-A	Persistence rate (%)
1	20	0	20	100
2	10	10	20	66.7
3	3	10	13	39.4
4	2	6.5	8.5	24.3
5	0	4.25	4.25	12.1
time event	**anti-B (N)**	**evanesced anti-B**	**Cumulative (non-evanesced) anti-B**	**Persistence rate (%)**
1	7	0	7	100
2	7	3.5	10.5	75.0
3	7	5.25	12.25	58.3
4	7	6.13	13.13	46.9
5	7	6.53	13.56	38.7

Fictitious example of a transfused cohort in which 35 patients formed alloantibodies against a moderately frequent antigen A, and a low-frequent antigen B. Allo-anti A and allo-anti-B both disappear at a rate of 50% per time period.

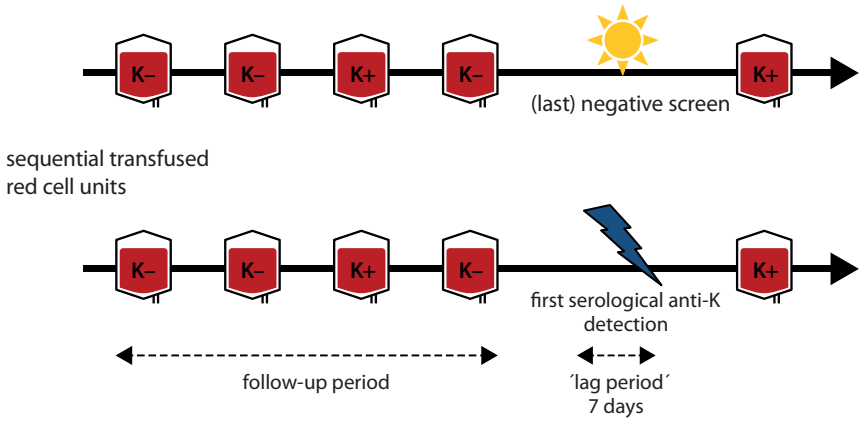
Due to a relatively high likelihood of (non-self) A exposure per time event, most of these anti-A's will be formed early. Thus, in this example, 88% of the 35 formed anti-A's will not be detected after a follow-up of 5 time events. In contrast, as anti-B forms at lower rates due to a lower chance of encountering this (non-self) antigen per time event, only 61% of the 35 formed anti-B's will not be detected after a follow-up of 5 time events.

Table S1 Overview of missing donor antigen data and the use of multiple imputation.

Antigen	Missing data (%)
D	0.0
C	0.5
C	0.4
E	0.4
E	0.5
C^w	44.7
K	0.5
Fy^a	35.0
Fy^b	45.8
Jk^a	19.3
Jk^b	19.9
Le^a	48.9
Le^b	57.7
Lu^a	78.5
Lu^b	90.6
M	26.5
N	38.0
S	21.5
S	38.7

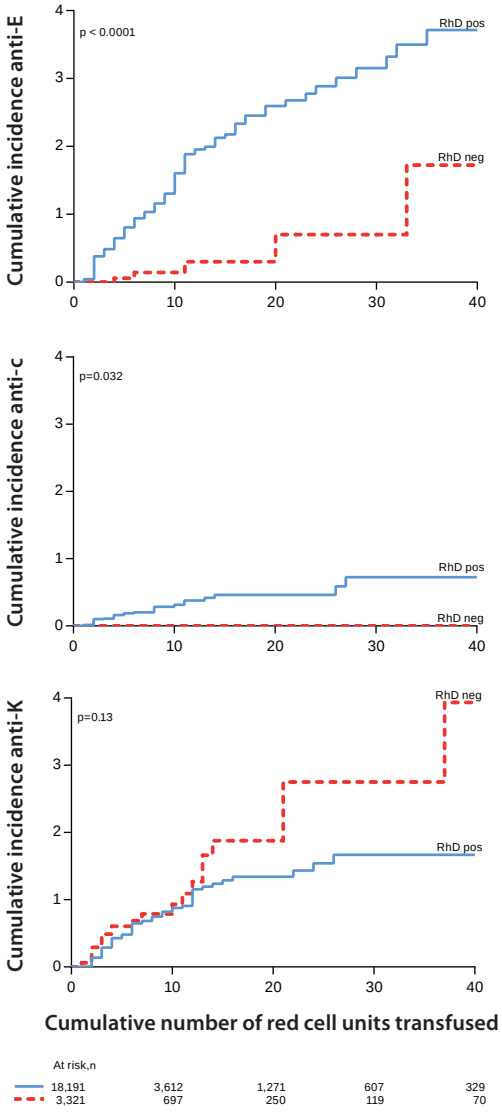
Percentages of missing antigen values in 152,412 red cell units transfused to 21,512 patients.
To address missing donor phenotypes, multiple imputation was used thereby creating five imputed datasets. Here, we assumed randomness of missing data (i.e missing values depended on observed data, but not on the value of the missing variable itself).[8,9] Imputation was only performed for antigen-negative cohorts with less than 50% missing antigen data. Consequently, Le^b, Lu^a, and Lu^b were excluded from antigen immunogenicity calculations.
Predictor variables for the imputation model included transfusion center, age under or above 45 years, sex, allo-immunization status, and known red cell antigen phenotypes of the blood product (i.e. other, non-missing antigens).

Figure S1 Illustration of calculations for cumulative numbers of transfused (antigen-mismatched) red cell units.



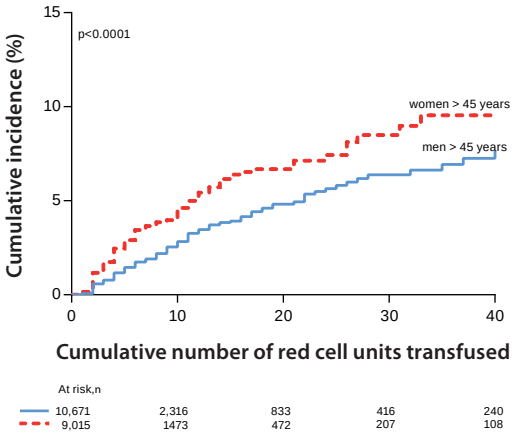
Non-alloimmunized patients were followed-up until their last available negative screen and alloimmunized patients were followed-up until the first positive screen. Alloimmunizations detected within seven days of a mismatched transfusion were excluded from analyses as they most likely represented boosting from previous induced alloimmunization rather than primary alloimmunization.
In this example, one non-immunized K-negative patient and one K-immunized patient both received four red cells units and one K-mismatched unit during their follow-up.

Figure S2 RhD-negative individuals do not form anti-c and rarely anti-E.



Due to linkage of the *RHD* and *RHCE* gene, approximately all RhD-negative patients express the c antigen and cannot form allo-anti-c. As only 2.9% of RhD-negative Dutch donors are E-positive, RhD matching strongly reduces the risk of allo-anti-E formation in RhD-negative patients. RhD phenotype is not associated to the risk of alloimmunization against other red cell antigens as here demonstrated for anti-K.

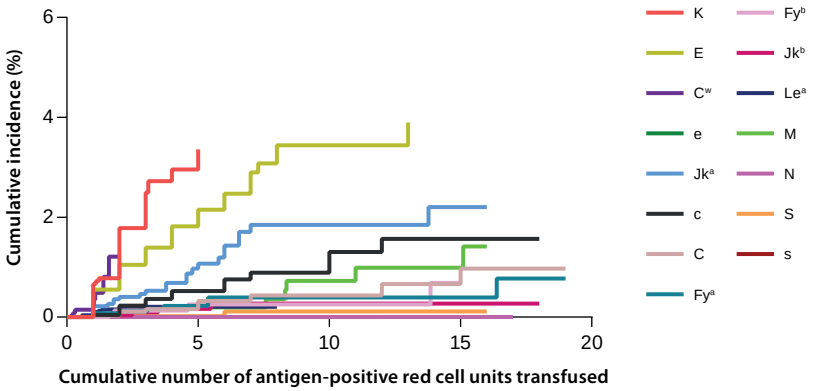
Figure S3 Cumulative alloimmunization incidences according to sex in patients aged 45 years and above.



	Cumulative alloimmunization incidences, % (CI):			
	against all antigens		against the antigens CcEe, C^w, and K	
Number of transfused units	Men >45 yrs N=10,671	Women >45 yrs N=9,015	Men >45 yrs N=10,671	Women >45 yrs N=9,015
5	1.41 (0.56-3.01)	2.91 (1.59-4.88)	0.88 (0.24-2.48)	2.24 (1.11-4.19)
10	2.73 (1.35-4.94)	4.62 (2.70-7.30)	1.85 (0.66-4.17)	3.46 (1.80-6.07)
20	4.60 (2.45-7.77)	6.69 (3.96-10.37)	3.52 (1.51-6.90)	5.00 (2.57-8.62)
40	7.08 (3.44-12.49)	9.51 (4.80-16.23)	5.23 (1.97-10.90)	7.91 (3.24-15.31)

Women as compared to men over 45 years of age showed statistically significant higher alloimmunization incidences (figure) due to higher Rh and K immunization rates (table).
CI = 95% confidence interval.

Figure S4 Cumulative alloimmunization incidences as a function of antigen exposure in the male population.



At risk, n

K	11,215	242	21	5	2
E	8,787	1,171	363	150	85
C^w	12,025	18	4	1	0
e	246	106	54	29	16
Jk^a	2,847	1,074	461	249	145
c	2,469	988	480	273	166
C	3,935	1,227	574	321	181
Fy^a	4,178	1,386	584	316	188
Fy^b	2,090	758	353	206	135
Jk^b	3,194	1,094	503	284	176
Le^a	9,571	664	156	52	18
M	2,709	1,029	465	245	148
N	3,432	1,124	491	261	159
S	5,520	1,316	512	247	134
s	1,347	542	259	159	105

	Male cohort Cumulative incidence, % (CI)				
Mismatched units (N)	**E**	**K**	**C^w**	**Fy^a**	**Jk^a**
1	0.55 (0.06-2.53)	0.66 (0.07-3.05)	0.24 (0.00-11.68)	0.08 (0.00-10.03)	0.22 (0.00-6.42)
2	1.05 (0.23-3.24)	1.78 (0.40-5.27)	1.21 (0.00-16.81)	0.12 (0.00-8.11)	0.41 (0.00-5.22)
5	2.15 (0.63-5.44)	3.36 (0.67-10.01)		0.23 (0.00-7.31)	1.07 (0.06-6.34)
10	3.44 (1.03-8.35)			0.39 (0.00-7.60)	1.43 (0.11-7.02)
15				0.39 (0.00-7.60)	1.85 (0.20-7.73)

The relative antigen immunogenicity and the antigen potency order observed in male did not differ from the entire cohort.
CI = 95% confidence interval. Only data from non-censored cohorts of at least 200 subjects are presented.

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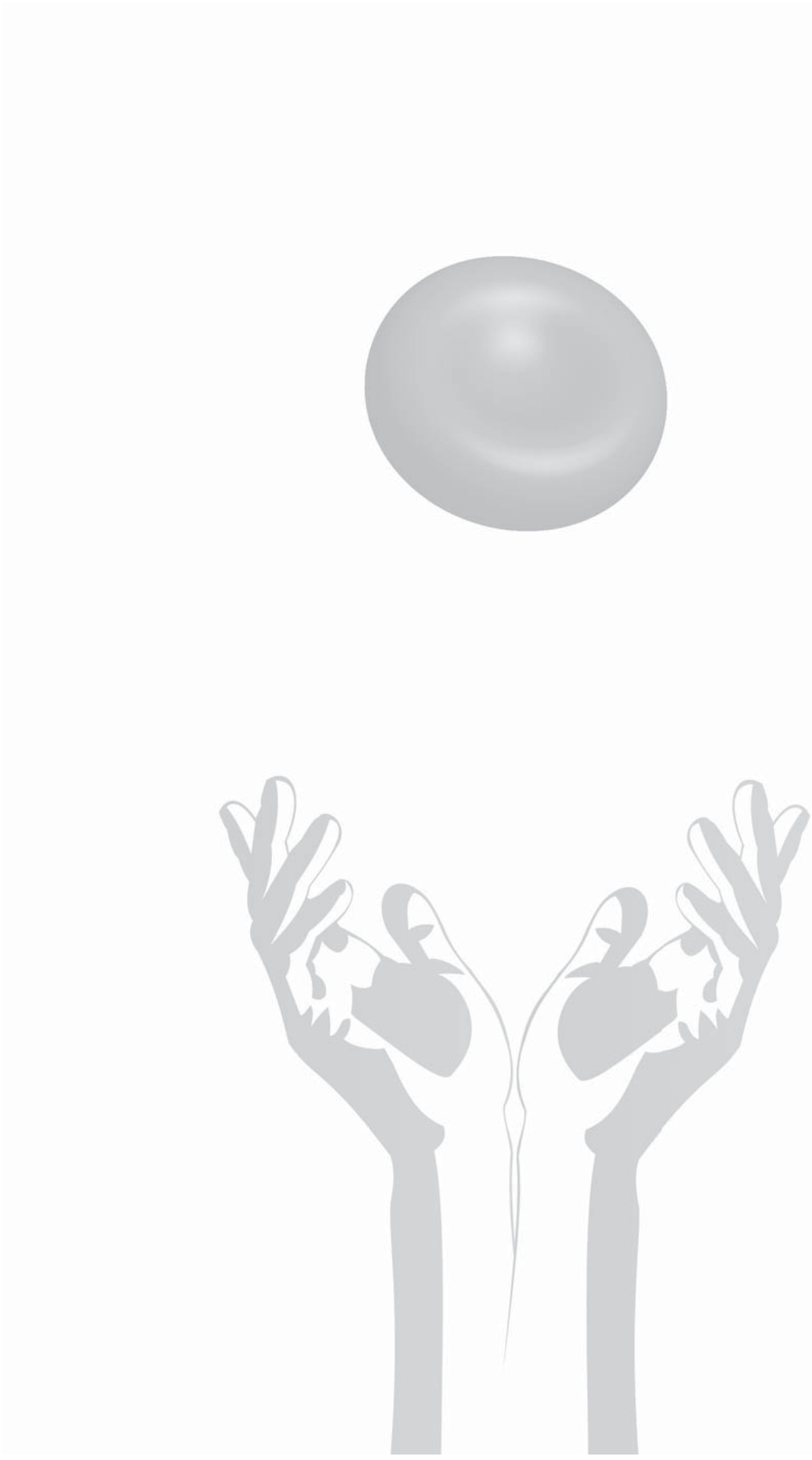

3

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.

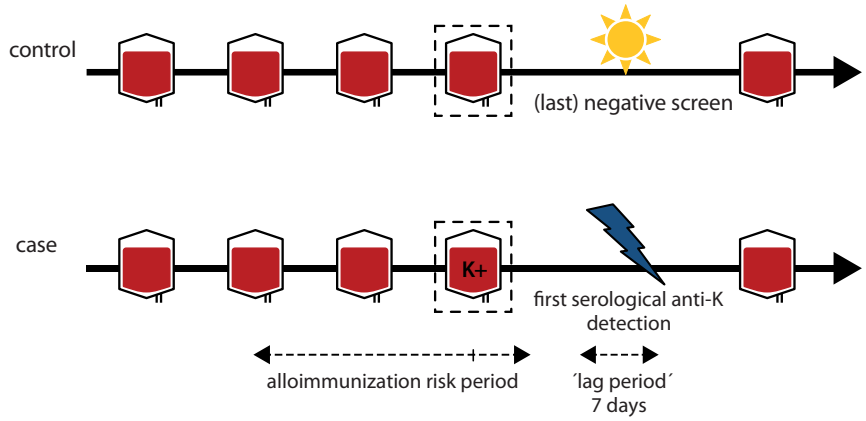
For all cases and controls, we recorded various clinical conditions during the alloimmunization risk period.

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.

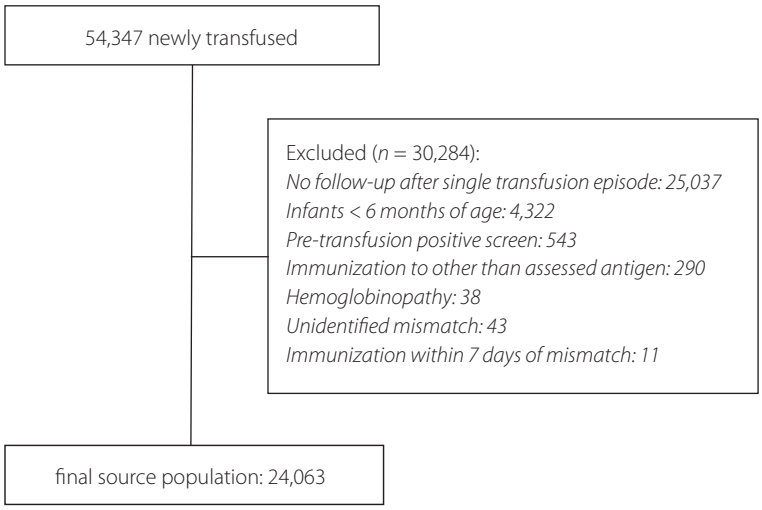


Figure adapted from: Evers, D., Middelburg, R.A., de Haas, M., Zalpuri, S., de Vooght, K. M., Visser, O., Péquériaux, N.C., Hudig, F., Schonewille, H., Zwaginga, J.J. Red cell alloimmunization in relation to antigens' exposure and their immunogenicity: a cohort study. *Lancet Haematol.* 2016;3(6):e284-92.

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

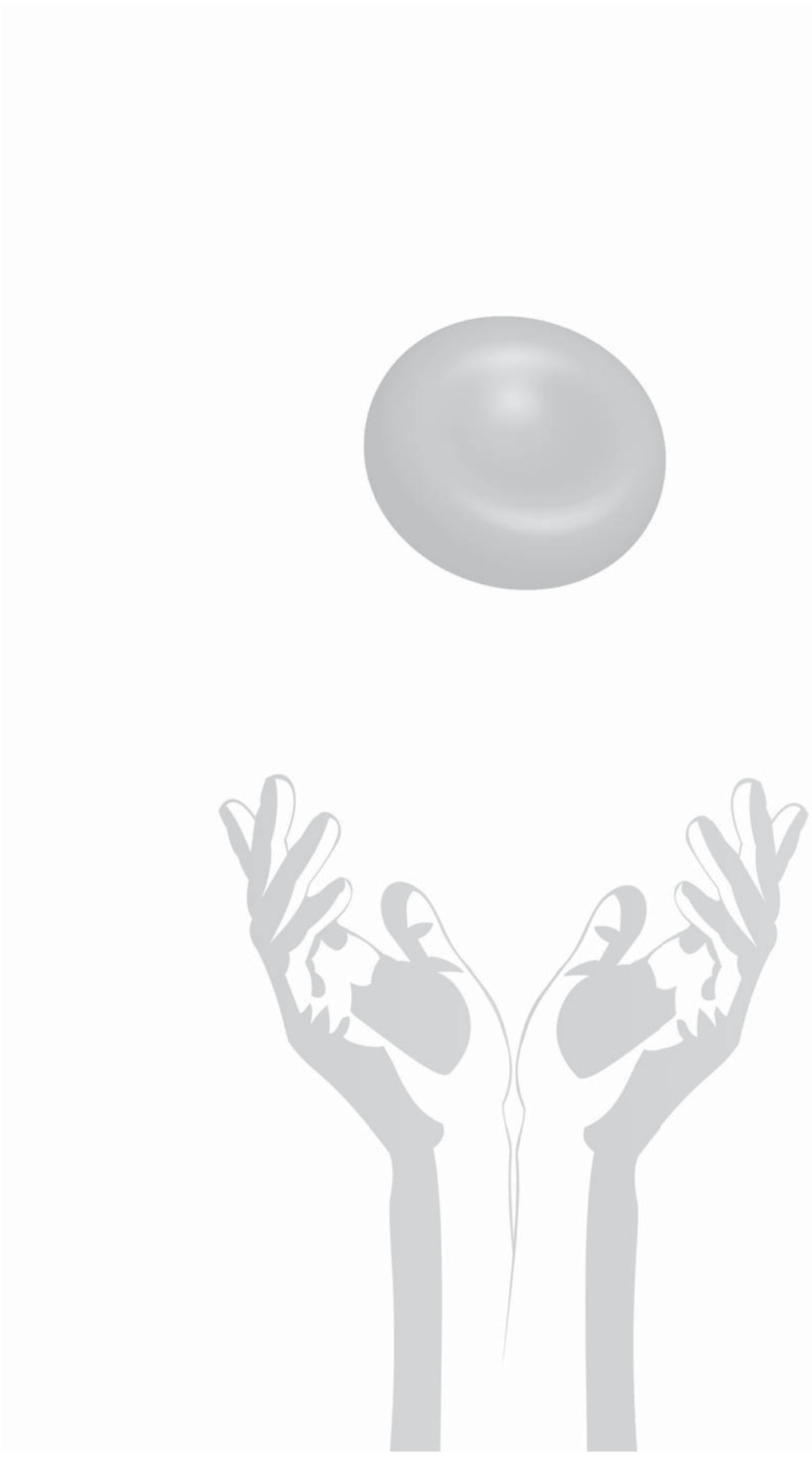
	Cases (N=505)	Controls (N=1,010)	RR (CI) *
Maximum leukocyte counts (x10^9/L) †			
4-10	140	249	*ref*
10-15	118	242	0.88 (0.56-1.20)
15-20	91	164	0.99 (0.70-1.39)
20-30	71	152	0.83 (0.56-1.21)
>30	43	96	0.79 (0.51-1.22)
Maximum CRP values (mg/L) ‡			
≤30	69	163	*ref*
30-100	95	184	1.20 (0.80-1.79)
100-200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



4

ABSENCE OF THE SPLEEN AND THE OCCURRENCE OF RED CELL ALLOIMMUNIZATION IN HUMANS

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With its unique anatomy and location amidst the circulatory system, the spleen allows an intimate contact between its resident cells and blood passing 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 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 thalassemic patients suggested splenectomy to be associated to increased red cell alloimmunization,[4,5] while others did not find any association.[6,7]

In the current study, we assessed the association between the anatomic absence of the spleen and (transfusion-related) red cell alloantibody induction in our multicenter case-control R-FACT study cohort. This cohort includes 505 alloimmunized cases and 1,010 non-alloimmunized matched controls among an earlier described, 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.[8] A detailed description of our case-control cohort and our used methodology has been published recently.[9]

Summarizing, 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. Here, 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 transfusion 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 abdominal surgery, while no patient underwent a splenectomy in the context of an autoimmune disease. Sixteen of the splenectomized

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	adrenectomy 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 respectively 23, 23, and 21 days after the first allo-M and allo-E exposure, the splenectomy, and the implicated transfusion.

patients received their implicated (*Nth*) transfusion at or after splenectomy (median 0, range 0-3612 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 (patient 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 until 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 which 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 (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 by 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, 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 splenectomized 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 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 not surprisingly 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]

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

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 approximate 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 (Δ p) 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).

Consequently, would splenectomy not have 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.

Patient	T1: number of red cell units received before splenectomy	T2: cumulative number of red cell units received up till last screen	pT1	pT2	Δ 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

Table 2 Continued.

Patient	T1: number of red cell units received before splenectomy	T2: cumulative number of red cell units received up till last screen	pT1	pT2	Δ p
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

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. Δ p = 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]

Contrary to our results, observational studies in patients with major thalassemia and sickle cell disease (a population not included in the current study) so far did not find any abrogation of red cell antibody development with splenectomy. Some even concluded these patients to be more prone to red cell alloimmunization.[4,5] Yet, hemoglobinopathy patients in need of splenectomy are often highly transfusion dependent, causing a beforehand 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 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 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 it of importance 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 independent 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 mediating 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 non-hemolytic 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 to mount a protective immune response following non-conjugated 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 to protection from primary red cell alloimmunization in the general transfused patient population.

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Table S1 Characteristics of 1,010 non-alloimmunized sampled controls during the alloimmunization risk period according to a history of splenectomy.

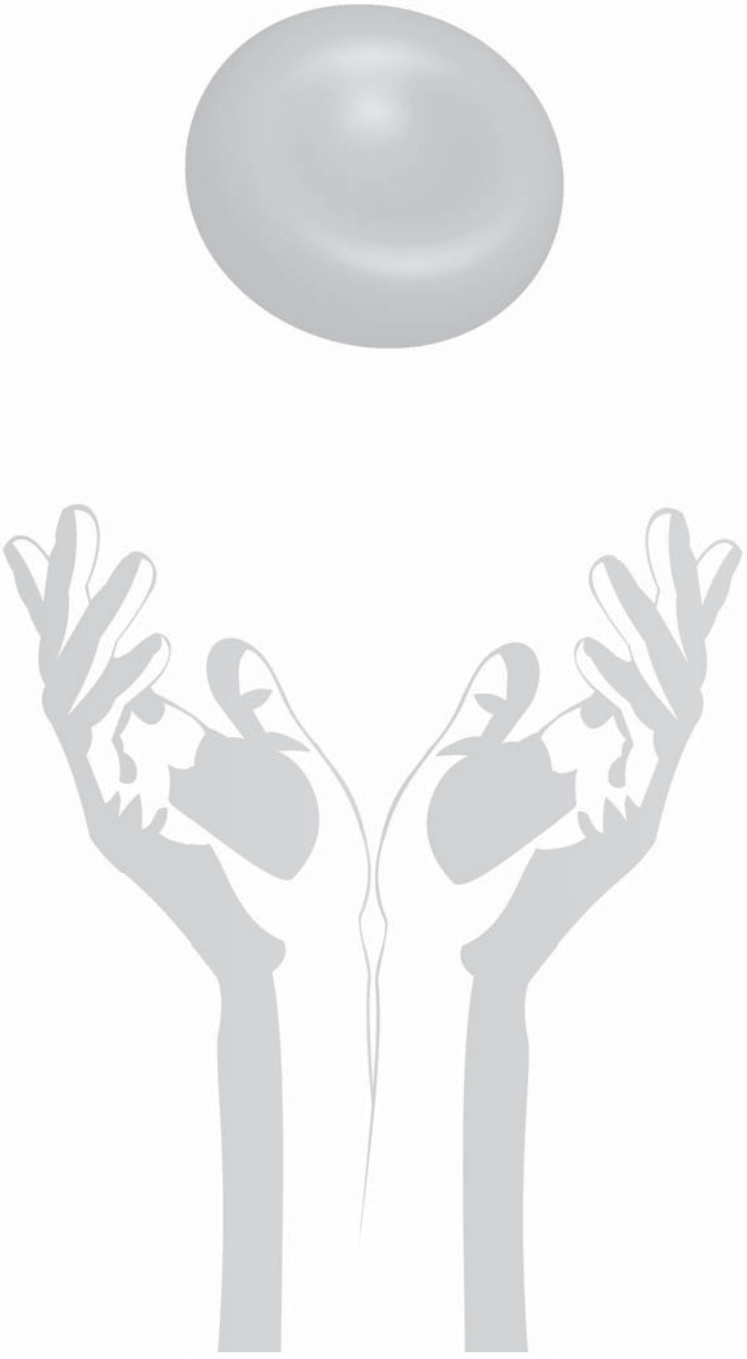
Characteristics	Splenectomized (N=19)	Non-splenectomized (N=991)	Missing	Possible confounder Y/N
General				
Men	12 (63.2)	556 (56.1)	0 (0)	Y
Age in years (median, IQR)	58.0 (38.5-74.3)	65.2 (51.9-75.1)	0 (0)	Y
Cumulative (lifetime) number of red cell units up till implicated transfusion (median, IQR)	4 (2-14)	4 (2-8)	0 (0)	NA
Diagnoses				
Atherosclerosis *	4 (21.1)	310 (31.3)	13 (1.3)	Y
Diabetes mellitus type 1	0 (0)	7 (0.7)	0 (0)	N
Diabetes mellitus type 2	4 (21.1)	172 (17.4)	1 (0.1)	Y
GFR ≤ 30 ml/min †	2 (10.5)	147 (14.8)	0 (0)	Y
Dialysis (chronic or acute) ‡	2 (10.5)	96 (9.7)	0 (0)	N
Chronic obstructive airway disease §	2 (10.5)	87 (8.8)	19 (1.9)	N
Organ transplant (in past or during risk period)	0 (0)	33 (3.3)	0 (0)	Y
Liver cirrhosis	0 (0)	24 (2.4)	2 (0.2)	N
Hematological malignancy	1 (5.3)	209 (21.1)	5 (0.5)	Y
Carcinoma	3 (15.8)	180 (18.2)	5 (0.5)	N
Sarcoma	3 (15.8)	15 (1.5)	1 (0.1)	Y
Interventions				
ICU admission days at ICU (median, IQR)	15 (78.9) 3 (1-8)	354 (35.7)	0 (0)	Y
Surgery thoracic, including CABG abdominal back or spinal cord	 3 (15.8) 17 (89.5) 1 (5.3)	 141 (14.2) 164 (16.5) 10 (1.0)	 0 (0) 0 (0) 0 (0)	 N Y Y

Immunosuppressant medication ||	9 (47.4)	414 (41.8)	13 (1.3)	Y
Chemotherapy	0 (0)	215 (21.7)	4 (0.4)	Y
Radiotherapy	0 (0)	37 (3.7)	0 (0)	Y
Hematopoietic stem cell transplantation (in past or during risk period) ¶	1 (5.3)	63 (6.4)	0 (0)	N
Treatment-related complications				
Leukopenia **	5 (26.3)	303 (30.6)	24 (2.4)	Y
Infections				
(tissue-invasive) severe bacterial ††	8 (42.1)	161 (16.2)	32 (3.2)	Y
Gram-positive bacteremia	1 (5.3)	82 (8.3)	7 (0.7)	Y
Gram-negative bacteremia	1 (5.3)	43 (4.3)	0 (0)	N
disseminated viral ‡‡	1 (5.3)	19 (1.9)	0 (0)	Y

Values are n (%), unless otherwise stated. Numbers of patients for whom data on certain conditions were not documented are presented as missing. Covariates were identified as a possible confounder of splenectomy when considered to be a potential risk factor for alloimmunization and being associated with splenectomy among the source population (i.e. represented by the sampled controls). An association with splenectomy was considered present in case of a ≥3% difference in covariate presence between controls exposed and controls.

IQR = interquartile range. NA = not applicable, as this represents a matched variable.

* systemic or coronary atherosclerosis. † 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. § chronic asthma bronchiale or chronic obstructive pulmonary disease. || medication under subcategory H02 (corticosteroids) or L04 (other immunosuppressants) within the World's Health Organization Anatomical Therapeutic Chemical (ATC) classification index. ¶ autologous or allogeneic. ** at least once measured leukocyte counts below lower limit of normal. †† defined as: abscesses, cardiac infections, infected foreign material, intra-abdominal infections, lower respiratory tract infections, meningitis, osteomyelitis, soft tissue infections, spondylodiscitis, upper urinary tract infections. ‡‡ defined as: viremia and varicella zoster infections.



5

IMMUNOSUPPRESSANTS AND ALLOIMMUNIZATION AGAINST RED BLOOD CELL TRANSFUSIONS

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Abstract

Introduction

Patients receiving red blood cell transfusions are at risk of developing alloantibodies against donor red cell antigens. The risk of alloimmunization is dependent on the number of units administered and the patient's genetic predispostion, but has also been suggested to be modulated by a patient's clinical profile. Our aim was to examine whether immunosuppressants suppress the development of clinically relevant red cell alloantibodies.

Methods

A two-center case- referent study was performed where case patients and control patients were sampled from all consecutive patients (N=17,750) who had received their first and subsequent red cell transfusions in a five year period in the study centers. Cases were all patients with a first detected red cell alloantibody preceded by negative antibody screens. Control patients were two-to-one matched to the case patients based on the number of red cell transfusions. Logistic regression analysis was used to examine the association between immunosuppressant exposure and the subsequent occurrence of red cell alloimmunization.

Results

A total of 156 case patients and 312 control patients in the study received a median of 6 transfusions (interquartile ranges 3-11). Among the total study population, 207 patients received immunosuppressive therapy, with 142 patients receiving only corticosteroids, 4 receiving only other immunosuppressants and 61 receiving both. The incidence of alloimmunization among patients using immunosuppressants was lower than among other patients receiving red blood cells, adjusted relative rate (RR) 0.55 (95% confidence interval (CI) 0.34- 0.91).

Interpretation

Our findings support a considerably lower risk of alloimmunization with the use of immunosuppressive medications.

Introduction

Patients receiving red blood cell transfusions are at risk of developing alloantibodies against donor red blood cell antigens.[1] Alloimmunization against clinically relevant red cell antigens can cause serious complications like acute and delayed hemolytic transfusion reactions. In light of this, it becomes important to study the risk factors associated with alloimmunization in detail, in order to predict which patients are most vulnerable to alloimmunization and may be considered for more extended matched red blood cell transfusions. On the other hand, identifying clinical factors protecting patients against alloimmunization would be equally important.

The risk of alloimmunization is dependent on the number of red cell units administered.[1] The extent of alloimmunization has been studied in various populations with the incidence of alloimmunization increasing with the number of units, ranging from 7% to 13% in a general transfused population.[1-2] The risk of alloimmunization is also determined by a patient's genetic predisposition to form an immune response to these non-self antigens.[3] In addition, it has been suggested that a patient's clinical condition is associated with modulation of the alloimmunization risk.[4] Immunosuppressive therapy could be of particular importance in this respect, because red blood cell transfusions and immunosuppressive therapy often coincide in intensive care, trauma, active autoimmune disorder, cancer, and organ transplant patients.

The use of immunosuppressants among a general transfused population and its effect on the risk of clinically relevant red cell alloimmunization, however, has not been reported and was the purpose of this study.

Methods

Design and study population

A matched case-referent study was performed at two Dutch university hospitals (Leiden University Medical Center, Leiden and University Medical Center Utrecht, Utrecht, the Netherlands). Details of our case-referent study design have been previously published[5] and are presented in chapter 3 of this thesis. In short, the source population comprised of all previously non-transfused, non-alloimmunized patients who received their first red cell transfusion at one of the study centers. The study period was January 2005 to December 2010 at Leiden University Medical Center and January 2006 to December 2011 at University Medical Center Utrecht, Utrecht.

Case patients were patients with first-time detected clinically relevant red cell alloantibodies and control patients were patients who did not have formed any clinically relevant red cell alloantibody after the same number of transfusions as the matched case. The control sampling was conducted on the principles of a risk-set sampling strategy,[6-7]

i.e. for any given case (with *N* red cell units received up until alloantibody formation), two control patients with at least the same number of units were randomly selected from the source population (figure 1). Control patients were then matched to case patients based on the *N* number of units received (figure 1). Case and control patients were also matched on the study center.

The transfusion policy in the study centers was as follows: 1. routinely transfused red cell concentrates were in SAGM and pre-storage leukoreduced and 2. all patients were routinely screened for alloantibodies before transfusion, which was repeated at least every 72 hour, if further transfusions were required.

Alloimmunization risk period

We first set out to define an 'alloimmunization risk period' preceding the antibody detection in order to identify the concurrent clinical conditions that in combination with an antigen mismatched transfused unit (implicated unit) could have led to alloimmunization.[5,8]
We measured all the study variables within this alloimmunization risk period.

This risk period stretched from 30 days before up to seven days after the implicated unit. We chose the risk period not to include the week just before the positive screen to permit at least one week to allow appropriate time for the development of alloantibodies (lag period). The risk period definition is illustrated in figure 1. A similar clinical risk period surrounding the *Nth* transfusion was defined for the matched control patients with the *Nth* transfusion corresponding to the implicated unit received by the case (figure 1).

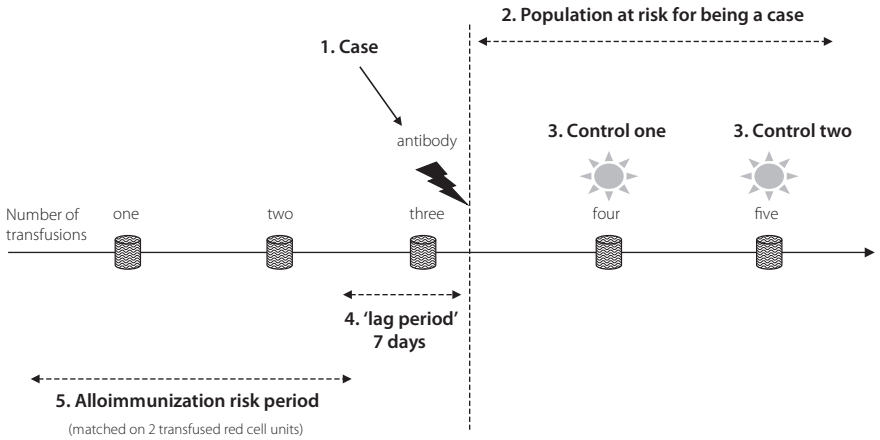
Using the above defined method to establish an alloimmunization risk period, we found in the majority (88%) of our case patients at least one transfusion with the mismatched antigen in the risk period immediately preceding the antibody identification. For the remainder of case patients, we looked further back into their transfusion history to identify the transfused unit with a mismatched antigen and re-defined the alloimmunization risk period as per the above mentioned definition around that particular mismatched transfusion.

Identification of initial (first time formed) clinically relevant red cell alloantibodies

Red cell alloantibodies were defined as warm reacting clinically significant antibodies (against: C, E, c, e, C^w, K, Fy^a, Fy^b, Jk^a, Jk^b, Le^a, Le^b, Lu^a, Lu^b, M, N, S and s), and were screened for using a three cell panel including an indirect antiglobulin test (LISS Diamed ID gel system) throughout the study period. Positive screening in the three cell panel led to subsequent identification of the antibody or antibodies by a standard 11 cell panel using the same technique.

Alloantibodies of other specificities than those mentioned, as well as cold reacting alloantibodies are not routinely detected by the three cell panel screening method and were thus not considered to be included as cases of clinical alloimmunization.

Figure 1 Control patient selection and the alloimmunization risk period.



The chronological order from case patient identification to alloimmunization risk period definition is marked from step 1 to 5.

1. A case is detected after three units of red cells received.
2. All transfusion recipients who received at least three units of red cells and developed no antibodies up until three transfusions are considered as the referent population.
3. From this referent population, two controls are selected at random.
4. A 'lag period' of seven days is introduced between the day of antibody detection to prevent the inclusion of patients demonstrating possible recall events. As such, the second but not the third unit transfused here might have mounted an alloimmune response and is defined as the implicated transfusion.
5. For both the case and the two matched controls, an alloimmunization risk period stretching from 30 days before up to 7 days after the second unit transfused is established as the alloimmunization risk period.

Medication classification

To classify the immunosuppressive therapy into corticosteroids and other immunosuppressants categories (table 1), the World Health Organization's ATC (Anatomical Therapeutic Chemical) classification index was used (source: http://www.whocc.no/atc_ddd_index). Medications classified under category H, subcategory H02 were included as corticosteroids; medications classified under category L, subcategory L04 were included as (other) immunosuppressants (table 1).

Data collection and definitions

Transfusion dates, results of the antibody investigations, patients' date of birth, gender, and clinical data on the presence of chronic obstructive pulmonary disease (COPD), infections (bacterial, viral, or fungal; infections diagnosed by laboratory serological techniques

including blood and tissue cultures), fever (temperature above 38.5 °C), transplants (organ and hematopoietic stem cell), allergies (food, dust, animal and chemical), autoimmune diseases, leukemia (acute lymphoblastic, acute myeloid, chronic lymphocytic, juvenile myelomonocytic), mature lymphoma, chemotherapy, surgeries (thoracic, abdominal, cranial and facial, upper and lower limbs excluding transluminal angiography), traumas (high impact traumas including cars, motorbikes and bicycles; falls) and diabetes (type 1 and type 2) were collected from clinical files within the defined alloimmunization risk period of alloimmunization. Use of immunosuppressive medications within this risk period was verified by consulting the hospitals' electronic patient dossiers and information management systems.

At the time of this analysis, we had not yet reached the target number of hospitals stated in the R-FACT protocol (500 case patients) due a general delay in initiating the R-FACT study protocol in other hospitals.

Data analyses

The association between the use of immunosuppressive medications and alloimmunization was modeled using a logistic regression model. Odds ratios were interpreted as relative rates throughout the manuscript. All relative rates (RR) were corrected for the matching factors (i.e. total number of transfusions and study center) and presented with a 95% confidence interval (CI).

We compared patients receiving 1. any immunosuppressive medication, 2. exclusively corticosteroids, 3. exclusively other immunosuppressants, and 4. both of these in combination, to patients not exposed to any of these medications, within the alloimmunization risk period.

The adjusted relative rates were adjusted for the above mentioned potential clinical confounders with age categorized as ≤25, 26-50, 50-75, and >75 years of age.

Results

Characteristics of the study population

Out of a total of 17,750 transfused patients, 468 patients were studied (156 case patients, 312 control patients). Fifty-six percent (N=261) of patients were from Utrecht and 44% (N=207) were from the Leiden study center. The study population had a median age of 59 years, (interquartile range (IQR) 38-70) and comprised of 56% males. Case patients had received a median of 6 units of red cells (IQR 3-11) before alloantibody formation. Antibodies were detected for the first time after a median of 123 days (IQR 25-333) following the first transfusion.

Use of immunosuppressive therapy in the alloimmunization risk period

A total of 207 patients used any immunosuppressant medications during the alloimmunization risk period including 54 cases (34.6%) and 153 controls (49.0%). Prednisone/ prednisolone (50.2%), dexamethasone (46.9%), hydrocortisone (24.1%), mycophenolate mofetil (17.9%) and cyclosporine (16.4%) were the most used immunosuppressants (Table 1). Information on medications and immunosuppressive therapy could not be traced for 18 patients (9 controls and 9 cases) and these patients were omitted from the analysis.

Table 1 Types of Immunosuppressive medication used by 207 out of 468 patients (44.2%) of the total study population.

Class and type	Number (%)
Corticosteroids	
Prednisolone/prednisone	104 (50.2)
Dexamethasone	97 (46.9)
Hydrocortisone	50 (24.1)
Methylprednisolone	34 (16.4)
Other	1 (0.5)
Other immunosuppressive medications	
Cyclosporine	34 (16.4)
Mycophenolate mofetil	37 (17.9)
Azathioprine	5 (2.4)
Antithymocyte globulin	9 (4.3)
Basiliximab	16 (7.7)
Tacrolimus	22 (10.6)
Thalidomide	3 (1.4)
Other	1 (0.5)

Among the source population, (i.e. represented by the control patients), patients using immunosuppressive medications were more often males, and younger as compared to patients not using immunosuppressive medications. Patients using immunosuppressive medications more often had (any type of) infection, allergies, leukemia, and mature lymphoma, more often underwent transplants, and more often used chemotherapy. They less frequently underwent surgeries and traumas as compared to patients not using immunosuppressive medications (Table 2). The distribution of auto-immune diseases, diabetes type 1 and type 2 was similar in both patient populations.

Table 2 Characteristics of 312 non-alloimmunized sampled controls during the alloimmunization period according to their exposure to immunosuppressive medications.

Characteristics, N (%)	None	Corticosteroids or other immuno-suppressant	Only corticosteroids	Only other immuno-suppressants	Corticosteroids and other immuno-suppressants
	(N=150)	(N=153)	(N=99)	(N=3)	(N=51)
Men	88 (58.7)	79 (51.6)	52 (52.5)	0 (0)	27 (52.9)
Age in years (median, IQR)	63 (53-75)	49 (31-65)	53 (37-70)	44 (26-44)	43 (28-59)
COPD	3 (2.0)	7 (4.6)	4 (4.0)	0 (0)	3 (5.8)
Infection*	36 (24.0)	56 (36.6)	31 (31.3)	1 (33.3)	24 (47.1)
Fever†	36 (24.0)	39 (25.5)	22 (22.2)	2 (66.7)	15 (29.4)
Transplants (organ and hematopoietic stem cell)	2 (1.3)	31 (20.3)	4 (4.0)	1 (33.3)	26 (51.0)
Allergies	7 (4.7)	12 (7.8)	3 (3.0)	1 (33.3)	8 (15.7)
Auto-immune diseases	4 (2.7)	4 (2.7)	4 (4.0)	0 (0)	0 (0)
Acute or chronic leukemia	11 (7.3)	25 (16.3)	14 (14.1)	2 (66.7)	9 (17.6)
Mature lymphoma	2 (1.3)	12 (7.8)	9 (9.1)	1 (33.3)	2 (3.9)
Chemotherapy	11 (7.3)	33 (21.6)	29 (29.3)	1 (33.3)	3 (5.9)
Surgeries	90 (60.0)	73 (47.7)	50 (50.5)	0 (0)	23 (45.1)
Trauma	16 (10.7)	4 (2.6)	4 (4.0)	0 (0)	0 (0)
Diabetes type 1	2 (1.3)	3 (2.0)	2 (2.0)	0 (0.)	1 (2.0)
Diabetes type 2	13 (8.7)	13 (8.5)	7 (7.1)	0 (0)	6 (11.8)

Values are n (%), unless otherwise stated. IQR = interquartile range. Values are n (%), unless otherwise stated. IQR = interquartile range. Information on immunosuppressive therapy could not be traced for 9 controls.

* includes bacterial, viral and fungal infections. † defined as temperature ≥ 38 °C at least once measured during the alloimmunization risk period.

Immunosuppressives and risk of alloimmunization

Table 3 presents relative rates for patients using any type of immunosuppressants, only corticosteroids, only other immunosuppressants or both, as compared to patients using none of these. Compared with patients not using any immunosuppressive medications, patients using only corticosteroids, only other immunosuppressants, or both all had a lower alloimmunization rate with an adjusted RR of 0.70 (CI 0.42-1.16), 0.51 (CI 0.04-7.10), and 0.19 (CI 0.07-0.53), respectively.

Table 3 Relative rate of alloimmunization in patients using only corticosteroids, only other immunosuppressants and both as compared to using none.

Type of immunosuppressant	Case patients	Control patients	Crude RR (CI) *	Adjusted RR (CI) †
None	96	150	*ref*	*ref*
Corticosteroids and/or immuno-suppressants	23	75	0.53 (0.34-0.81)	0.55 (0.34-0.91)
Only corticosteroids	43	99	0.68 (0.43-1.08)	0.70 (0.42-1.16)
Only immunosuppressant	1	3	0.45 (0.04-5.00)	0.51 (0.04-7.10)
Corticosteroids and immuno-suppressants	10	51	0.28 (0.13-0.59)	0.19 (0.07-0.53)

RR = relative risk. CI = 95% confidence interval.
* adjusted for the matching variables (number of matched transfusions and hospital).
† adjusted for matching variables, sex, age, COPD, infection, fever, transplants, allergies, auto-immune diseases, leukemia, mature lymphoma, chemotherapy, surgeries, trauma, diabetes type 1, and diabetes type 2.

Discussion

In our case-referent study among previously non-transfused, non-alloimmunized patients, exposure to immunosuppressives was associated with a lower incidence of clinically relevant red cell alloantibodies against donor red blood cells.

The number of patients using *only* other immunosuppressants was very low and hence, RRs presented with wide CIs. These low patient numbers reflect the standard clinical practice where immunosuppressive therapy frequently encompasses prednisone or other corticosteroids.

To appreciate our findings, several aspects need to be discussed. Strength of our study is the control sampling strategy. By using a risk-set sampling strategy, our control patients formed a representative sample of the source population.[7] In this study we

examined the combined immune modulating effects of transfusion exposure and that of immunosuppressives administered in the defined alloimmunization risk period. For this purpose, we carefully defined this risk period aiming to be able to study clinical concurrent events with possible immune modulating effects. While the observed protective association between immunosuppressive therapy and alloimmunization may in part be the result of other risk factors for alloimmunization that are also associated with the use of immunosuppressants (confounding factors), we carefully measured other risk factors and adjusted for them in our analyses.

Although the possibility of unknown transfusions at a different hospital cannot be entirely ruled out by our strategy due to absence of such information in the transfusion records of the study centers, all selected patients needed to have a negative antibody screen preceding the first transfusion and at least followed by one post transfusion antibody screen. This strategy is not entirely excluding recall immune responses to earlier primary immunizations. We, however, do not expect this to have affected our study findings as there is no reason to believe that patients with unknown previous transfusions or with unknown previous antibodies are more likely to be exposed (or unexposed) to any of the potential confounding variables.

To our knowledge, this is the first study in humans that shows the presence and extent of the protective effect of immune suppressive medications on alloimmunization against clinically relevant red cell antigens. A causal nature of the observed association with use of immunosuppressants is biologically plausible. Their role in suppressing transplant rejection in patients undergoing solid organ transplants has been well documented.[9] In addition, immunosuppressive therapy has been shown to impair humoral immune responses to vaccines and antigens.[10-11] With respect to corticosteroids, hydrocortisone has been shown to diminish *in vitro* responses to streptokinase-streptodornase and tetanus toxoid vaccinations as indication of a suppressed immune response.[12] This diminished immune response in the presence of corticosteroids has been attributed to transient lymphocytopenia by the redistribution of circulating T cells to other body compartments.[13] It has been also demonstrated that proliferation of T cells can be inhibited by corticosteroids.[14-19] For example, glucocorticoids inhibit production of T cell growth factor and block the clonal expansion necessary to amplify a primary response.[17,20,21]

Other immunosuppressive drugs also suppress T cell responses.[22] Proliferation of B and T lymphocytes is inhibited by immunosuppressants like mycophenolate and rituximab,[11,23]while agents like cyclosporine and tacrolimus inhibit the activation and differentiation of T cells by inhibiting calcineurin. In addition, a lower influenza vaccine antibody response and diminished T cell proliferation responses have been shown with these drugs in immunosuppressed liver transplant patients.[24]

Considering the mechanisms of alloimmunization against red cell antigens, this process is both B cell and T helper cell dependent. Although the short lived formation of non-naturally occurring IgM antibodies by IgM B cell memory cells is mainly T cell independent,

the subsequent memory B cell response and the formation of more high affinity IgG is T cell helper dependent. It is therefore likely that in the presence of corticosteroids and other immunosuppressive drugs, the T cell mediated responses to donor red cell antigens are impaired. Of course, the observed mediated risk reduction of alloimmunization need not be entirely caused by immunosuppressive agents, however, a direct attributive effect is strongly plausible.

As such, when aiming for an eventual alloimmunization risk prediction on the basis of clinical factors, immunosuppressives might be added to such a prediction score. This may enable to distinguish high risk patients for alloimmunization who might benefit from cost effective, extended donor blood phenotype matching strategies.

In summary, corticosteroids and other immunosuppressant medications appear to have a considerable protective effect on alloimmunization in patients transfused with donor red blood cells. While immune activating conditions are often the reason to start these drugs and coincide with their use, the inhibiting effect that was observed in our studies might be even an underestimation of the true effect of these drugs on the alloimmunization response.

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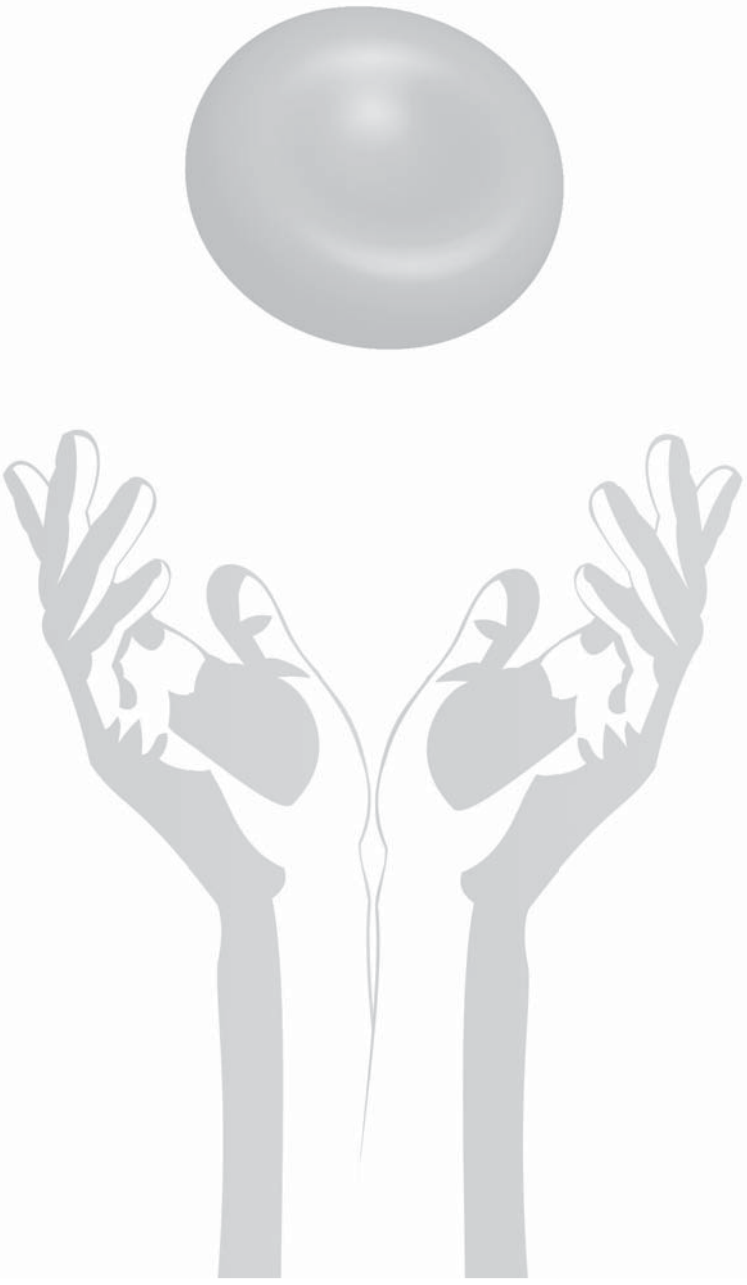

6

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.

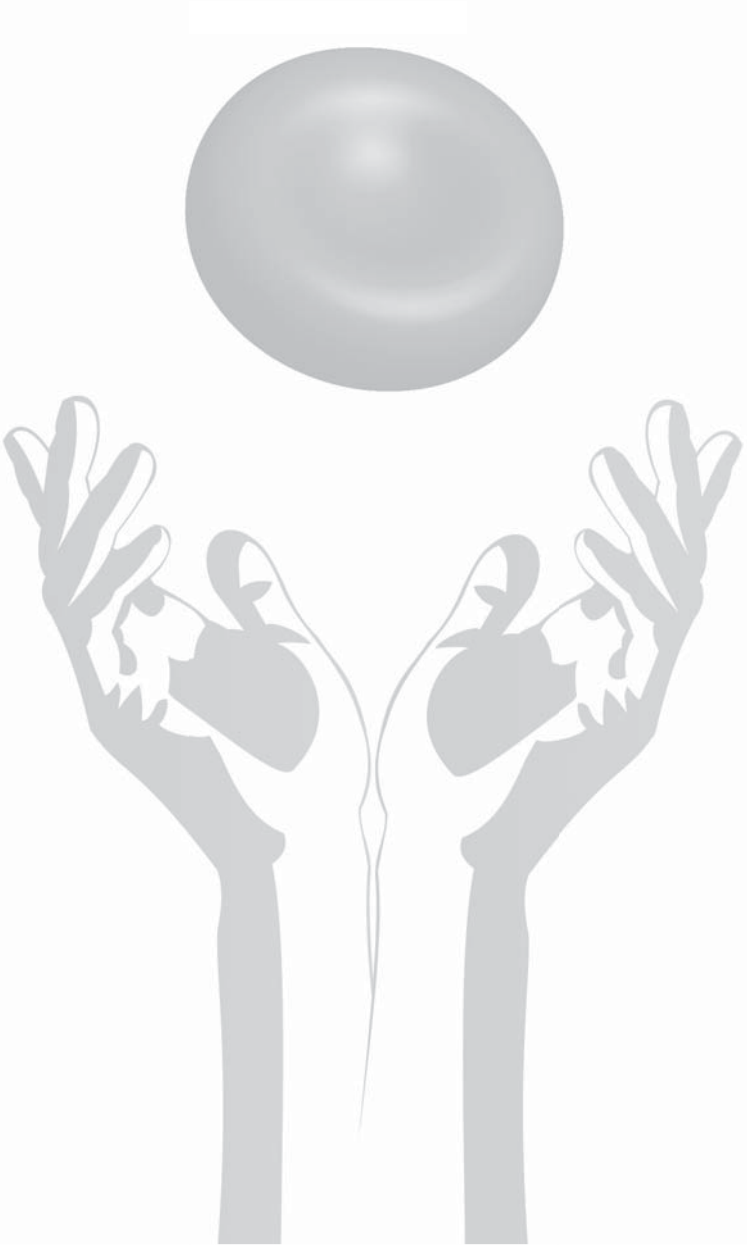
Determinant	Confounders
All	Age, gender, (duration of) ICU admittance, thoracic surgery, atherosclerosis, GFR ≤ 30 ml/min, dialysis.
Acute leukemia	Idem as under 'all', plus: abdominal surgery, DM2, COPD, MPN, lymphoma, carcinoma
Myelodysplastic syndrome	Idem as under 'all', plus: abdominal surgery, 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).



7

SUMMARY AND FUTURE PERSPECTIVES

Summary and future perspectives

In this thesis, we report on various determinants which we found associated with red cell alloimmunization in humans, with the eventual aim to reduce red cell alloimmunization and its potentially detrimental consequences by risk factor based matching strategies. Here, we first highlight the identified risk factors against the background of former evidence. Finally, we discuss future research perspectives.

Optimizing red cell antigen matching: critical antigens

Antibody formation to red cell alloantigens requires exposure to alloantigens together with a certain activation of the recipient's humoral immune system. The reported alloimmunization prevalences are surprisingly variable ranging from only 3% to as high as 58%.[1-9] These wide ranges likely reflect the differences in study designs and selected patients, e.g. inclusion of previously transfused and thus more exposed patients, inclusion of previously alloimmunized patients, and the length of serological follow-up. Our strategy of use of an incident new-user cohort enables estimation of the incidences of alloimmunization as a function of exposure within a cohort of transfusion-naive patients. With this approach, our group previously managed to confirm the intuitive assumption that the risk to develop alloantibodies increases with the transfusion burden.[10]

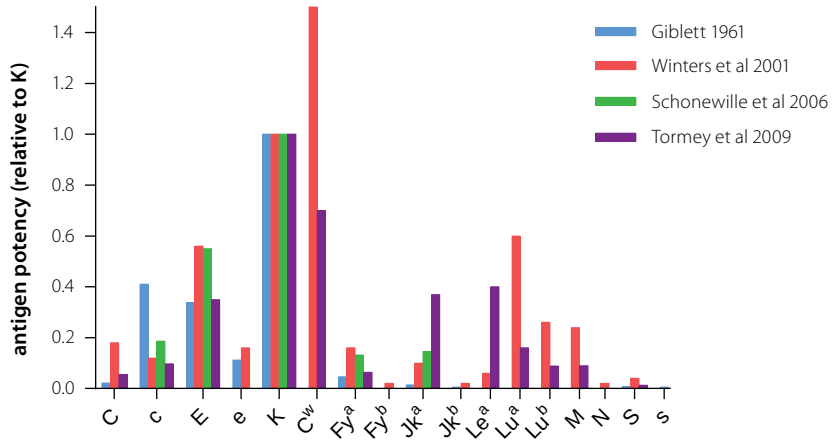
Expansion of our cohort from two to six participating hospitals allowed us to assess antigen-specific alloimmunization incidences and with it the exposure corrected immunogenicity of these antigens. In **chapter 2**, we illustrate that anti-E, anti-K, anti-Jk^a, and anti-c are the most prevalent formed alloantibodies among the 7.7% of transfused patients who formed alloantibodies after having received at least 40 units of red cells. With a policy of serological matching against their cognate antigens, the population would have benefited from a 74% reduction of red cell alloimmunization.

Considering prevention by matching for certain antigens, it is important to realize that antigens are not equally distributed nor are they equally immunogenic. Several studies reported on antigen immunogenicity estimates over the past decades.[11-14] Likely related to the often used 'Giblett-equation', these studies yielded conflicting conclusions regarding the potency of all antigens, except for K (Figure 1). Giblett-based calculations deduce immunogenicity estimates from prevalence figures by comparing the observed numbers of antigen-specific antibodies with the calculated probability of non-self antigen exposure.

However, several factors potentially influencing the results obtained by this equation need to be taken in consideration:

First, the Giblett equation is based on average antigen frequencies in a donor and recipient population of Caucasian origin and assumes that the chance of alloimmunization

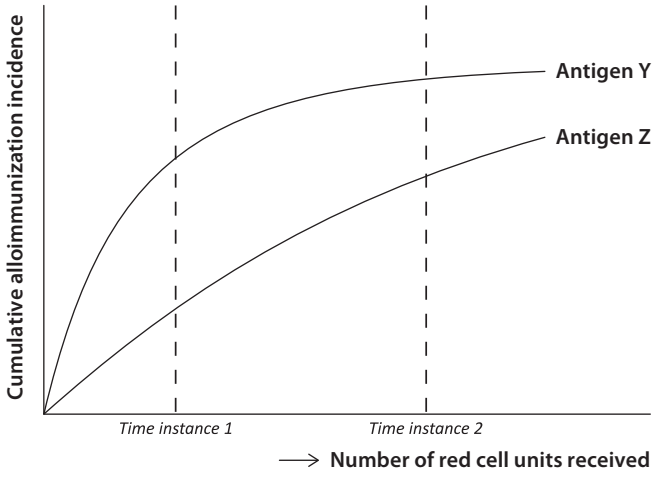
Figure 1 Summary of previously estimates of antigen immunogenicity with K as reference.



is linearly increasing with antigen exposure. However, it seems more likely that when one has not formed an alloantibody after multiply mismatched units, the likelihood to do so after the next mismatched unit will be even smaller because of the recipient being a so called 'non-responder' patient. Accordingly, most hemophilia patients receiving prophylactic clotting factor infusions form inhibitors to these products early after initiation of regular suppletion.[15] Consequently, for an antigen with moderate frequency (e.g. Jk^a), the cumulative incidence curve rises relatively early during accumulation of red cell transfusions and then flattens after *N* red cell transfusions, because patients lacking expression of this antigen will reasonably be fastly exposed and immunized. Contrary, for an antigen with low frequency (e.g. K) the initial increase of the incidence curve will be slower because, although most red cell recipients do not express the antigen themselves, this also applies to the donor population. Thus, the odds of encountering non-self K as compared to non-self Jk^a per transfused red cell unit are far lower. Figure 2 illustrates this exposure-related flattening for a fictitious antigen Y with moderate frequency and an antigen Z with low frequency. At time point 1, the number of patients who have formed anti-Y far exceeds those who have formed anti-Z, while at time point 2 these numbers approximate one another. Ultimately, prevalence-based immunogenicity estimates derived from the Giblett equation will induce an overestimation of the relative immunogenicity of low-frequent antigens (e.g. K), especially in a multiply transfused population.

A second important caveat in assessing antigen immunogenicity concerns current RhD matching strategies. In fact, the likelihood to be exposed to the C, c, E, and e antigens is determined by the influence of *RHD* and *RHCE* gene-linkage. That is, as only 6% of the

Figure 2 The estimated relative immunogenicity of antigens is dependent on exposure.



As fictitious antigen Z represents a low-frequent antigen as compared to the fictitious moderate frequent antigen Y, most responsive patients will have been exposed and thus have formed anti-Y at time point 1, while primary exposure still occurs after time point 1 for antigen Z. Consequently, the four-fold higher estimated potency of antigen Y as compared to antigen Z at time point 1 decreases to a factor 2 at time point 2.

Caucasian RhD-negative population express the E antigen,[16] RhD-negative patients receiving only RhD negative red cell products are rarely exposed to E. Conversely, as 67% of RhD-positive individuals lack E,[16] 23% of RhD-positive patients are at risk of E alloimmunization with routine RhD matching. None of the reported prevalence-based calculations accounted for this gene-linkage. Consequently, when matching for one antigen indirectly also involves matching for another (gene-linked) antigen, inaccurate estimations of antigen exposure, and thus of the antigens' immune system stimulating potencies, will be made.

Third, prevalence-based calculations estimate the antigen's potency relative to another (e.g. relative to K), however, do not inform about absolute risks according to alloantigen exposure. The latter, however, will be specifically decisive when debating extended matching for the individual patient.

Finally, previously reported studies did not consider higher immunization risks due to intrinsic antigen differences between e.g. Caucasian donors and recipients from non-Caucasian origin (e.g. hemoglobinopathy patients), neither considered the lower risk of some patients due to receiving extended matched products (e.g. auto-immunized or

previously alloimmunized patients or women in the reproductive age). As the odds for antigen exposure in all these patient populations differs from that in the general, non-extended matched Caucasian patient population, earlier presented immunogenicity calculations represent a mixture of these risks.

Considering the above, we limited our incident new-user cohort to primarily Caucasian red blood cell recipients, while we plotted the incidence of antigen-specific alloimmunization as a function of exposure to all units received and second to only all antigen-positive units received by all antigen-negative patients. The latter enabled us to deduce unbiased relative antigen immunogenicities from these incidences. With this approach, we confirm in **chapter 2** that K indeed is the most immunogenic antigen, followed by E, C^w, e, Jk^a, and c. Based on these data, prophylactic extended matching for the K, E, e, Jk^a, and c antigens would prevent 74% of primary alloimmunization events. While K, E, e, and c matching is often attempted for in high risk patients, prevention of anti-Jk^a is currently not aimed for in developed countries.[17, 18] Yet, our results underline the importance of Jk^a matching as this antigen is shown to be highly potent in eliciting an antibody response with the antibody known to easily induce complement mediated hemolysis. Although the observed high immunogenicity of C^w might come as a surprise, we do not recommend to implement extended matching for this antigen. The chance of subsequent exposure after primary immunization is only 2% per transfused unit[16] and, more importantly, severe hemolysis by anti-C^w is rare.[19-21]

Finally, we have to realize that even with complete (molecular) typing of red cell recipients, it is unlikely to find a completely matched red cell unit for all patients due to limited donor resources.[12] Furthermore, matching logistics will be even more compromised in countries with less organized blood collecting services as compared to the Netherlands, as well as in case of red cell recipients with a different ethnic background from the donor population, and in situations of acute need of blood and related lack of time e.g. acute trauma.

Notwithstanding the above, the in our studies provided knowledge on the potency of several red cell antigens hopefully enables clinicians and blood bankers to prioritize which blood group antigens should be primarily matched between donor and recipient.

Optimizing red cell antigen matching: identifying the critical patient population

Next to exposure to high immunogenic non-self red cell antigens, alloimmunization requires a recipient´s immune system to be capable of mounting a significant adaptive immune response upon exposure. Currently, there is a limited understanding of what factors dictate and which immune cells and signals are essential to this specific immune response in humans. In this light, the factors we found associated with alloimmunization risk need to be placed into the complex string of events leading to alloimmunization.

Inflammation as a modulator of alloimmunization

Contrasting the processing of most microbial organisms in lymph nodes, senescent and damaged red cells are primarily sequestered in the red pulp of the spleen. Under non-inflammatory circumstances, splenic macrophages play a major role in clearing these cells from the circulation via their SIRPα interacting with CD47 on red cells.[22-25] Lower levels of clearance seem to take place in the liver, while essentially no clearance occurs in peripheral lymph nodes.[23] Mice experiments showed that in the absence of inflammation these animals only rarely form red cell alloantibodies following red cell transfusions,[26] possibly related to the functioning of red pulp macrophages during these circumstances. Indeed, red pulp macrophages have been implicated in both the induction of regulatory T cells and the inhibition of $CD4^+$ T cell responses.[24] As red pulp macrophages are important for clearing ageing autologous hematopoietic cells, they are rightly situated to have a regulatory function protecting against harmful autoimmune responses.

It is generally believed that for a more effective adaptive immune response, antigenic stimulation needs to be accompanied by additional, often cytokine mediated, 'danger signals' originating from e.g. pathogen-activated innate immune cells.[27-29] In line with this, we observed an association between a patient's inflammatory condition due to infection at the time of red cell transfusion and the development of red cell alloantibodies. In **chapter 3**, we demonstrate alloimmunization risk to be modulated by the type of infection, its intensity, and the patient's inflammatory response. In detail, patients with severe (tissue-invasive) bacterial and viral infections demonstrated higher alloimmunization incidences as compared to the general transfused patient population. In intriguing contrast, blood-borne infections with Gram-negative bacteria (known to express LPS on the outer surface of their cell membrane) coincided with an almost 2-fold reduction of alloimmunization risk. The available evidence supports the following hypotheses on the underlying immunological mechanisms:

First, in murine red cell transfusion models, exposure to several pro-inflammatory pathogen-associated molecular patterns (PAMPs) such as poly(I:C) and hypomethylated CpG-containing bacterial DNA unequivocally promoted red cell alloimmune responses.[23, 26, 30-32] Of interest, not all inflammatory triggers unanimously enhance alloantibody production, as lipopolysaccharide (LPS) pretreatment was associated with substantially suppressed alloimmunization rates in mice.[31]

These sharp contrasting outcomes on red cell alloantibody production with mono-administration of poly(I:C) versus LPS in mice underline that, within a similar inflammatory clinical phenotype, different intracellular signalling cascades and specific gene expression profiles can be activated depending on the specific interaction with their receptors on innate immune cells (pattern recognition receptors, PRRs). [33, 34] With a cell specific distribution of Toll-like receptors (TLRs),[33, 34] it seems reasonable to argue that a specific type of innate stimulus evokes a specific innate immune response.

Currently, dendritic cells (DCs) have been shown to be key players in the process of red cell alloimmunization as they are the important drivers of $CD4^+$ T cell responses. Following LPS administration, impairment of in vivo $CD4^+$ T cell proliferation via malfunctioning of a specific type of splenic conventional DCs (named "bridging channel $CD8^-$ $33D1^+$ DCs") was identified to underlie diminished alloimmunization with allogeneic red cells transfused.[27] The authors ascribed this phenomenon to a LPS-induced preactivation of these DCs leading to a lost capacity to present red cell alloantigens. Yet, in light of the above mentioned experiments, one might question whether and how these tolerance inducing mechanisms are trigger-specific, i.e. LPS causing a restraint of conventional DC maturation and skewing red blood clearance towards the macrophages, while poly(I:C) and CpG predominantly trigger DC activation.

A second interesting suggestion can be derived from etiologic mechanisms of some autoimmune diseases in which immune activation due to antigenic mimicry by several microorganism has been postulated. [35-37] Similarly, in experimental models, previous exposure to microbial T cell epitopes via a pathogen with small peptide homology to red cell antigens was demonstrated to significantly enhance primary alloantibody responses.[38] Whether potential existing similar mimicry between certain bacterial or viral epitopes could have played a causal role in our observations, can yet not be substantiated. However, the distribution of alloantibodies known to also occur 'naturally' (i.e. supposedly originating from red cell antigen-microbe mimicry) did not differ between patients with and without severe bacterial infections, disseminated viral infections, and Gram-negative bacteremia, suggesting an at most minor influence of mimicry.

Finally, we should consider our results to be at least partly influenced by the clinical conditions and treatments that necessarily follow the specific infections we studied. These sequel mediated associations may have been missed and as such not included in our multivariate regression analyses. In this respect, various antibiotics, including some types of cephalosporins commonly used for the treatment of Gram-negative bacterial infections, are known to suppress mitogenic responses of B and T lymphocytes.[39-41] Similarly, severely septic patients in addition to antibiotics often receive corticosteroids to diminish the potential harmful effects of extensive cytokine release. In consistency with our findings reported in **chapter 5**, such treatments likely also attenuate the alloimmune response in patients with Gram-negative sepsis.

Unlike murine ´single-stimulus´ experiments, real-life microbial infections in humans thus bring along exposure to a spectrum of simultaneous modulators. In addition to the treatments given, one microorganism may contain various components of which some might suppress (e.g. bacterial lipopeptides and LPS) and others might stimulate (e.g. hypomethylated CpG-containing bacterial DNA) adaptive immune responses.[42, 43] As such, it might well be possible that the presence of two different species, although belonging to the same microorganism family (e.g. Gram-negative enterobacteriae), disparately affect red cell alloimmunization. Unfortunately, the size of the current study

limits us to properly evaluate this. So far, except for one study demonstrating non-significant enhancement of red cell alloimmunization in polyoma virus infected mice,[38] the immune modulating potential of individual microorganism species with regard to red cell alloimmunization has not been assessed. Hopefully in the future, larger data sets could further detail and substantiate the in **chapter 3** observed associations between types of infections and red cell alloimmunization.

The spleen's critical role

As mentioned earlier, the spleen´s unique anatomy and location amidst the circulatory system allows an intimate contact between its resident immune cells and blood cells passing this organ.[24, 44, 45] The spleen is a preferential site for follicular B cell maturation and critical for the survival of IgM memory B cells, the latter being a unique B cell population in the marginal zone of the spleen producing natural IgM antibodies to e.g. polysaccharide structures.[46-49] In asplenic patients, IgM memory B cells are absent and these patients are at increased susceptibility to encapsulated bacterial infections.

Although T cell dependent B cell responses are generally preserved following splenectomy,[46] the spleen has been shown pivotal for antibody production against both autologous and allogeneic hematopoietic cell antigens.[50-55] Even in the presence of pro-inflammatory poly(I:C) stimulation, splenectomized mice showed a substantially attenuated antibody production against allogeneic red cells,[23] which at least was due to an impairment of priming and proliferation of antigen-specific $CD4^+$ T cells outside the splenic microenvironment.[27, 52, 55] So far not evaluated, splenectomy may also result into a restraint of B cell priming together with red cells shunting away towards the highly tolerogenic hepatic compartment.[55, 56] As such, removal of the spleen is used as a beneficial treatment for steroid-refractory autoimmune-mediated thrombocytopenia (ITP) and anemia (AIHA).[57-59]

In **chapter 4**, we evaluated the role of the spleen and, more specific, a history of splenectomy in transfusion-induced primary red cell alloimmunization. Alloimmunization following splenectomy was a highly unlikely event (relative risk (RR) 0.04, CI 0.01-0.55). Only one patient among an estimated number of 443 splenectomized patients (0.23%) developed red cell alloantibodies upon subsequent red cell transfusions, which is in sharp contrast with the 2.1% alloimmunization prevalence mentioned in **chapter 2**. Intriguing, splenectomy did not prevent the induction of an anti-M antibody, implicating a maintained IgM memory B cell response in this single patient. Thus, although of substantial influence, splenectomy is here demonstrated not to completely abrogate red cell alloimmune responses. We hypothesize some remaining splenic tissue or previously immunized B cells transferred via a concomitant transplantation of a solid organ to account for this single immunization.

Our results seem in contradiction with the few published cross-sectional studies in thalassemic and sickle cell patients of which some reported splenectomy to be associated

with antibody induction and others did not find any association.[60-62] None so far observed an abrogation of red cell alloantibody induction following splenectomy. However, one should recognize that thalassemic patients in need of splenectomy are often highly transfusion dependent. As most of these former studies did not correct for the cumulative numbers of red cells units received at the time of primary alloimmunization, potential exposure-related confounding should be considered. In addition, incomplete reporting of data, such as the receipt of extended matched and/or leukoreduced blood by some but not all analyzed patients, may have further compromised the validity of these studies. Finally, none of these studies comment on the timing of splenectomy in relation to the primary alloimmunization. Possibly, some of the splenectomized patients were already alloimmunized before this surgical intervention. As such, we cannot exclude that the different results and conclusions of these earlier studies as compared to our study are to be explained by the presence of (indication) bias..

Following our results, concerns for red cell alloantibody development in anatomic asplenic patients who are in need of high numbers of red cell transfusions seem unnecessary. As such, they do not need products matched beyond ABO/RhD. Future studies will need to (re-)evaluate whether this conclusion can also be extended to other asplenic patient populations e.g. patients with functional hyposplenism associated to celiac disease, autoimmune rheumatic disease, or caused by vaso-occlusive sickle cell disease crises.

Treatment-related immunosuppression

Finally, in **chapter 5 and 6**, we illustrate the strong protective role of immunosuppressive therapy in general. First, patients using corticosteroids and/or other immunosuppressive agents demonstrated a two-fold decreased risk of red cell alloimmunization (RR 0.55, CI 0.34-0.91). Second, patients with acute leukemia (either of myeloid or lymphoblastic origin), with mature (B or T cell) lymphomas, or patients post-autologous or -allogeneic stem cell transplantation, demonstrated a three-fold decreased incidence of clinically relevant antibodies against red cell alloantigens, which could similarly be ascribed to immunosuppressive (chemo-/ immuno)therapy.

These are the first large studies to support decreased alloimmunization risk in immunocompromised patients. As such, although not coming to a surprise, they are of importance to the transfusion medicine field.

Contrasting our results, previous studies concluded oncologic patients to have a similar or even an increased alloimmunization risk as compared to the general transfused population.[4, 7, 9, 63, 64] However, as the likelihood that one has formed alloantibodies increases with the number of exposures, patients who have formed alloantibodies will in general have been exposed to a higher number of red cell transfusions as compared to non-alloimmunized patients. These earlier studies thus roughly compared high-intensively transfused patients with less intensively transfused patients. As illustrated in **chapter 2**,

such an analysis will without doubt reveal an existing association of red cell alloimmunization with diseases that are in general supported with intensive red cell transfusions. Indeed, myelodysplastic syndrome (MDS) has so far been defined as a risk factor for alloimmunization and matching for high immunogenic antigens in this patient population reduces alloimmunization.[65] Yet, the observed association between MDS and red cell alloimmunization seems not due to intrinsic characteristics of the disease as we here demonstrate, but is primarily explained by the fact that MDS patients are often transfusion dependent and, consequently, exposed to a much higher transfusion burden. Thus, MDS patients not receiving treatment have a similar red cell alloimmunization risk *per transfusion event*, and are even protected from alloimmunization during treatment with chemotherapeutic agents. Thus, in general, one should take into account both the expected cumulative exposure as well as current treatments with immunosuppressive agents with regard to matching strategies for the individual hemato-oncological patient. Indeed, a recent study did not find any benefit of additional Rh/K matching in patients with acute leukemia and lymphoma as hardly any patient formed antibodies,[65] likely related to their immunosuppressive condition.

Our RRs do not take into account the dosing of and duration of immunosuppressive treatments, and whether or not agents were received as part of a combination regimen. It is rational, however, to regard patients receiving multiple dose-intensive immunosuppressive agents as more likely unresponsive to red cell alloantigen exposure. Also, patients at advanced stage of treatment i.e. patients still under treatment and already having received a large number of immunosuppressive treatments, might be considered more immunosuppressed as compared to patients who just initiated their treatment course. In line, following chemo- and/or immunotherapy for malignancies, the immune system remains dysfunctional for a certain period of time, depending on the intensity of the received treatment.[66-69]

Our findings support the notion that dose-intensive immunosuppressive therapy is the principal determinant of alloimmunization as non-treated patients with acute leukemia and mature lymphoma showed similar alloimmunization incidences to patients without these disease entities. However, we cannot exclude non-measured confounders associated with the likelihood of not receiving treatment (e.g. co-morbidities and disease stage) to have counteracted diminished immune responses. Intriguing, but only of speculative nature, the observed effects could be partly due to a direct interplay between the tumor and cells of the immune system. This process of host immune system subversion is a common hallmark of both hematological and solid tumors. Inflammatory signals from malignant cells initiate the recruitment of immune suppressor cells such as myeloid-derived suppressor cells and Foxp3 expressing regulatory T cells. Additionally, the production of effector cell suppressing cytokines (e.g. IL-10, TGF-β, and TNF-α), and polarization from a T helper 1 towards a T helper 2 response consequently result into the establishment of a tumor tolerant microenvironment.[70-74] If this mechanism would attribute to the observed

diminished alloimmunization incidences in patients with hematological malignancies, one would expect that especially patients with advanced stage of disease independent of the receipt of treatment would be protected from red cell alloimmunization. Unfortunately, the patient numbers in this study were too small to discriminate the alloimmunization risk per stage of disease.

Optimizing red cell antigen matching: future perspectives

The ultimate goal of the ongoing R-FACT study is to eventually establish an accurate alloimmunization prediction model, thereby enabling practical and risk-based clinical decision on extensive matching. In this perspective, the identified determinants of red cell alloimmunization (whether associated to induction or protection) serve as an important start for such a model. Yet, continuing research is needed to advance our understanding of immunobiological process of red cell alloimmunization, and identify other relevant determinants of this process. Ultimately, our efforts should lead to a prospective study on the feasibility and efficiency of extended matching for high-risk patients, with risk classifications based on the here and in future to be identified determinants.

Nature and Nurture

Future research focusing on other clinical determinants of alloimmunization should emphasize on 'nature' and 'nurture' as patient-based modulators of red cell alloimmunization.

Regarding nurture, it has repeatedly been suggested that certain environmental factors skew adaptive immune responses. For example, (early) exposure to bacterial commensals and helminthes infections have been implicated to modulate the immune system towards protection against various autoimmune diseases such as type 1 diabetes mellitus by selectively modulating the T helper 2 response and driving the regulatory arm of the immune system.[75-77] The latter also seems to explain the low propensity to develop allergic disorders observed in helminth-infected cohorts.[78] Vice versa, the dramatic increase in atopic diseases in the developed world might be a direct consequence of the eradication of helminth infections.[75] Thus, growing up in rural areas or keeping domesticated animals during early childhood (both associated with high microbial burden exposure), one's dietary contents (associated to the biomass and diversity of gut microbiota),[79] and use of antibiotics among other factors may all modulate the patient's response to allogeneic red cell antigens, similarly as they do for chronic inflammatory disorders.[80-82]

Next, taking a patient's genetic constitution ('nature') into consideration when deciding on matching seems another interesting approach. Although the costs and logistic challenges of high-throughput genotyping of blood group systems are still high, these tools will likely become available on a more routine base. Thus, genetic risk factor screening could

in future be easily added to these blood group gene arrays.[83] As we more extensively discussed in **chapter 1**, only a minor fraction of the genetic basis of red cell alloimmunization so far has been elucidated, with the majority of studies merely having focused on HLA gene polymorphism associations.[84-90] Knowledge of this topic can be extended by learning from related diseases e.g. evidence on the polygenic nature of inhibitor formation upon factor VIII administration in hemophilia A patients. This disease and its treatment has several features in common with red cell transfusions e.g. administration of the product via the blood stream, a risk to induce alloantibody formation upon exposure, and (at least in a subgroup of patients) the administration of a human-derived product. Over the last decade, the hemophilia research field has made several steps forward by linking a large number of single nucleotide polymorphisms (SNPs) in immunomodulating genes to inhibitor formation including SNPs in the CTLA-4, tumor necrosis factor (TNF)α, and interleukin-10 (IL-10) genes. [91-97] Second, a genome-wide association study on RhD allo-immunization by Sanquin Research in collaboration with Cambridge University just recently finished its sampling of DNA material from over 2,000 pregnant women and will soon start its analysis.[98] Obviously, results from this study might be translatable to allo-immunization to other red cell antigens and should help design future genetic research on transfusion-induced red cell alloimmunization.

Consistent with research on hemophilia A, studies performed in patients vaccinated against hepatitis B, measles, and influenza have demonstrated that variations in genes controlling adaptive immunity may predict vaccine efficiency.[99, 100] Although targeting a different antigenic processing pathway (due to their non-intravenous administration and a common use of conjugates or other adjuvants), microbial vaccines similarly to red cell alloimmunization aim to affect T cell dependent adaptive immunity. As such, the genetic background of vaccine non-responders might overlap with patients not forming red cell alloantibodies despite repeated allogeneic red cell exposure. In this regard, meta-analyses for hepatitis B responses found evidence that variants in class II HLA and IL-4 were significantly associated with humoral immune responses.[100-103] Other studies suggested associations with SNPs in cytokine genes, cytokine receptor genes, and toll-like receptor (TLR) genes. A comprehensive overview of these studies has been recently published by Newport et al.[99]

With the human genome sequence being completely elucidated and current techniques enabling high-throughput genome-wide analyses, it now seems feasible to extrapolate the above mentioned evidence into a large scale case-control study on genetic risk factors that modulate red cell alloimmunization. In this regard, we are currently planning to further expand our ongoing R-FACT study by prospectively sampling patient material at the time of red cell alloantibody detection from both antibody responders and non-responders. Subsequent analyses on immunomodulating genes should at least focus on polymorphisms in HLA class I and II genes (preferentially in relation to antigen-specific alloimmunization), and genes related to immune cell signaling e.g. chemo- and

cytokines and their receptors, toll-like receptors, molecules involved in costimulation, and nuclear transcription factors.

In addition to the above mentioned nurture and nature associated risk factors, biomarkers of the immune status of the patient may be predictive of red cell alloimmunization. In a first (retrospective) analysis on this subject which we discussed in **chapter 3**, we did not observe any association of the level of leukocytosis and CRP values as markers of inflammation with alloimmunization, possibly due to the multifactorial nature of these parameters. Other immune markers such as quantity and functionality of B, helper T, and regulatory T cell subsets might be better discriminative. Yet, such an analysis requires a complex study encompassing a substantially large cohort of patients being sampled at fixed time points over a considerable period of follow-up.

Finally, IgG immune responses to childhood vaccinations, as well as (age-adjusted) titers of naturally occurring IgM antibodies against antigen A and antigen B may predict the response to allogeneic donor red cells. Especially the latter seems intriguing as these IgM responses represent T cell independent immune responses.[104] Interestingly, the spleen and its proper functioning seems essential both for induction of T cell independent memory B cell responses [44, 105, 106] as well as IgG responses to red cell allogens (as discussed in **chapter 6**).

Benefits of matching

The studies presented in this thesis have tried to find an answer on questions starting with 'who' and 'what'. Who deserves to receive extensively matched donor red cell units? What red cell antigens should be taken into consideration when deciding to transfuse extended matched donor red cells?

The 'why' question has not received much attention so far. Yet, it is the driving force behind our studies.

Extensive matching in patients with sickle cell disease and thalassemia has proven to be effective, although most studies did not directly compare extended with non-extended matched patients.[61, 107] Preemptive extended matching for selected antigens (here: matched for the antigens c, C, E, K, Fy^a, Jk^a, and S) as compared to merely ABO/RhD matching reduced the primary alloimmunization rate by 5.3% (8.1% versus 2.9%) in a cohort of patients undergoing elective (cardiac) surgery.[108] In a post hoc analysis on patients who received merely red cell units, this absolute risk difference increased further to 8.0% (9.4% versus 1.4%; confidence interval (CI) 0.4-16.0). Indeed, patients who received platelets next to extended matched red cell units had comparable alloimmunization rates as compared to those receiving ABO/RhD matched units as they developed (non-D) Rh and K antibodies after cognate antigen exposure through platelet transfusions. Therefore, the few residual antigen incompatible red cells in platelet products can counteract the potential effect of extended red blood cell matching with regard to red cell alloimmunization prevention. Future studies should explore whether less antigen exposure by single donor

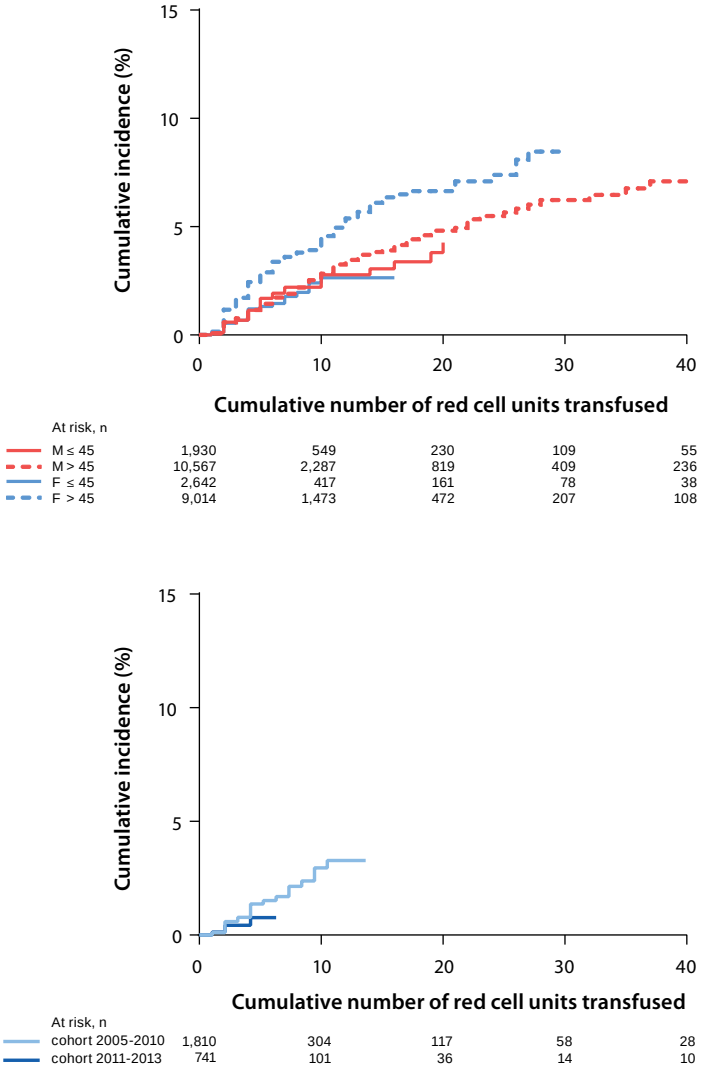
platelet apheresis products or even Rh/K matching with platelet transfusions could further reduce alloimmunization. Due to the low red cell antigenic load with platelet transfusions, we, however, estimate that the additive risk of non-matched platelet transfusions is negligible both for low immunogenic antigens and for the immunocompromised patient population.

In most cases, transfusions can be matched to existing antibodies thereby preventing antibody-antigen interactions and thus hemolytic transfusion reactions. Pregnancies, however, may be severely complicated by earlier alloimmunization due to ongoing maternal antigen exposure by the fetus. This especially accounts for earlier developed anti-K and anti-c.[109] For this reason, matching beyond ABO and RhD in women of childbearing age is nowadays routine practice in most European countries. In the Netherlands, women under 45 years of age receive K, and since 2011 additionally c and E, matched blood.[17] Although several reports have demonstrated an increased risk of fetal hemolytic complications for red cell alloimmunized pregnant women,[109, 110] no studies so far have assessed the beneficial effects of the introduction of K, c, and E matching in young women on alloimmunization incidences and its clinical consequences.

In this respect and in addition to our data reported in **chapter 2**, we observed significantly lower cumulative alloimmunization incidences among women under 45 years of age as compared to women above 45 years of age (4.4%, (CI 0.2-20.5) and 9.5% (CI 4.8-16.2) after 40 units received, log-rank p 0.013, Figure 3 upper panel). These differences seemed solely due to decreased Rh/K alloimmunizations in young women, since alloimmunization rates to non-Rh/non-K antigens did not differ between women under and above 45 years of age (Table 1). Furthermore, men under and above 45 years of age demonstrated similar alloimmunization incidences, excluding age as an explanatory factor. Of importance for accurate interpretation, the majority of the young women in our cohort received K, but not c and E, matched blood as this latter matching practice has only been nationwide established since 2011. Indeed, the 10 immunizations against c and E which we observed in women under 45 years of age all occurred before the introduction of matching for these antigens. In contrast, only one single anti-K immunization event occurred.

A preliminary analysis, which needs to be consolidated by extended follow-up, suggests a significant effect intensification from additional c and E matching (Figure 3, bottom panel). Only 4 of 741 (0.54%) women under 45 years of age consecutively transfused from January 2011 onwards as compared to 27 of 1,810 (1.49%) receiving red cell units between 2005 and 2010 developed alloantibodies. Due to the short follow-up and the consequently small cohort of women who received both K, c and E matched blood, differences fail to reach statistical significance (log-rank p 0.08). Notwithstanding the latter caveat, these findings strongly suggest that matching for E and c in addition to K is substantially effective in reducing alloimmunization rates.

Figure 3 Additional matching for K, c, and E protects against alloimmunization.



Upper panel: cumulative incidences of red cell alloimmunization according to age and sex. Lower panel: cumulative incidences of red cell alloimmunization in women under 45 years matched for K (cohort 2005-2010), and in addition also to c, and E (cohort 2011-2013). Only data from non-censored cohorts of at least 200 subjects are presented.

Additional antibody formation

Although the work presented in this thesis focuses on primary alloimmunization against a single antigen, it is well known that previously auto- or alloimmunized patients are at increased risk of developing additional antibodies with subsequent transfusions.[111, 112] As such, avoidance of exposure to antigens to which immunization has already occurred in addition to avoidance of exposure to high immunogenic antigens, is currently aimed for in these patients.

The risk to subsequently develop additional alloantibodies after primary immunization is patient-specific, i.e. resulting from a so-called high-responder phenotype to which nature and nurture associated factors contribute.[113, 114] In addition, immune activation by existing red cell alloantibodies themselves might play a role. In this regard, an immune response elicited to a particular high immunogenic antigen might enhance the response to weaker antigens. One potential mechanism, closely related to the earlier mentioned "non-exofacial polymorphic structures" (NEP) hypothesis (see **chapter 1**),[115] involves epitope spreading. Here, (an epitope of) antigen X appears in the context of a HLA class II molecule carried by a naive B cell as a result of this cell's phagocytic ability. When the B cell receptor (BCR) on this B cell is specific for antigen Y, subsequent activation of this B cell by a $CD4^+$ T cell which is sensitized against antigen X will lead to production of antibodies specific for antigen Y. Thus, immunization against antigen X may induce immunization against antigen Y.

Alternately, existing antibodies of IgG class can also suppress rather than enhance an immune response to non-cognate antigens.[116, 117] Here, phagocytosis of IgG opsonized allogeneic red cells via Fcγ receptors results into a rapid clearance of these cells from the circulation, thus preventing B cells from binding to other non-self red cell antigens. Additionally, IgG opsonized red cells may elicit inhibitory FcγRIIB signaling in B cells and as such prevent B cell activation. The earliest evidence for such an existing 'antibody-mediated immune suppression' (AMIS) was provided by the observation that ABO incompatibility between mother and child affords a degree of protection against Rh hemolytic disease of the fetus and newborn, because of anti-A or anti-B antibodies destroying the fetal red cells in the maternal circulation before immune system recognition.[118] Similarly, next to protecting against RhD immunization, prophylactic anti-D administration to women bearing a RhD-positive child was recently associated to an additional significant decreased risk to develop anti-E (personal communication, Zwiers, Koelewijn, van der Schoot et al, manuscript in preparation).

Current evidence of observational studies so far does not substantiate either epitope spreading nor AMIS to dominate immune responses in case of existing non-RhD antibodies. In this regard, in one study the type of first formed antibodies appeared not to be associated with the probability and type of the subsequent alloimmunization.[119] In agreement, we observed similar cumulative alloimmunization incidences to the non-matched non-Rh/K antigens in women under 45 years as compared to the rest of our incident new-user cohort (Table 1).

Table 1 Alloimmunizations to non-Rh/ K according to gender and age.

Number of transfused units	Men ≤45 yrs N=1,826	Men >45 yrs N=10,671	Women ≤45 yrs N=2,551	Women >45 yrs N=9,015
2	0.11 (0.00-18.19)	0.20 (0.01-1.82)	0.37 (0.00-5.47)	0.27 (0.01-2.02)
5	0.58 (0.00-9.00)	0.57 (0.08-2.39)	0.82 (0.02-6.59)	0.66 (0.09-2.75)
10	0.91 (0.01-9.76)	0.99 (0.20-3.22)	1.07 (0.02-8.89)	1.14 (0.18-4.22)
20	0.91 (0.01-9.76)	1.34 (0.23-4.66)	1.07 (0.02-8.89)	1.71 (0.26-6.21)
40	1.41 (0.01-15.87)	1.97 (0.30-7.07)	1.76 (0.01-18.31)	1.71 (0.26-6.21)

Women under 45 years of age demonstrated similar alloimmunization rates to non-Rh/K antigens as compared to older women.

Nevertheless, prevention of primary alloimmunization is of upmost importance both in a setting of epitope spreading as well as with AMIS. In case of high clinical relevance of epitope spreading, further prevention of alloimmunization (i.e. after a first antibody has already formed) would not suffice. Instead, absolute prevention of ($CD4^+$ T cell) sensitization, by primary avoidance of all or at least most allogeneic antigens would avoid both primary and subsequent additional immunization. In case the concept of protective AMIS proves to also exist beyond RhD antibodies, secondary matching to prevent (further) alloimmunization seems less needed. Again, primary matching for at least all high immunogenic antigens should deserve our main emphasis.

Yet, only a subset of the patient population can practically receive extended matched products due to financial costs and logistic feasibility, hereby once more underlining the high importance of accurate identification of the high-responder patient and high-risk conditions for alloimmunization. Accordingly, the studies and their outcomes presented in this thesis will serve future tailoring of preventive matching strategies by having identified respectively exposure to certain high immunogenic antigens, an infectious-disease related inflammatory condition, a treatment induced state of immunosuppression, and a functioning spleen to be ultimate determinants of red cell alloimmunization.

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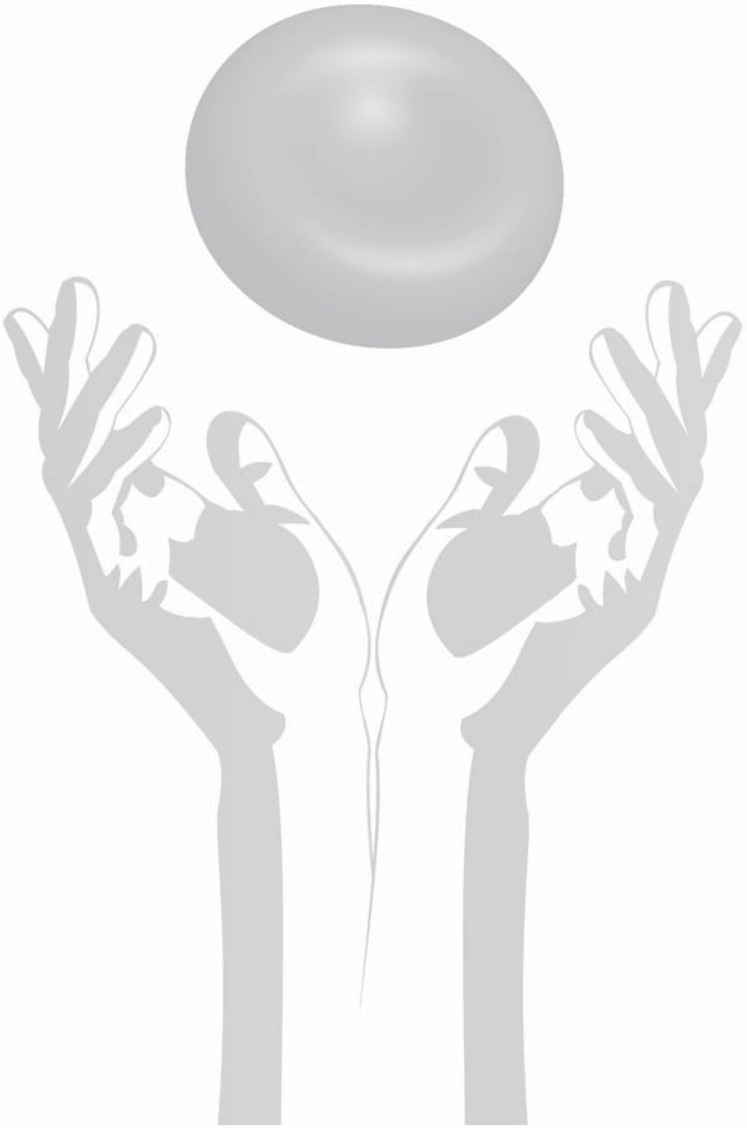


8

NEDERLANDSE SAMENVATTING

In Nederland doneren jaarlijks meer dan 300.000 donoren bloed. Rode bloedcel transfusies vormen een essentieel onderdeel van de behandeling van patiënten bij wie de aanmaak van bloed gecompromitteerd is of bij wie sprake is van anemie door acuut of chronisch bloedverlies.

Rode bloedcellen bevatten op de buitenmembraan van de cel diverse eiwit-, vet- en suikerstructuren. Deze structuren kunnen tussen individuen onderling in geringe mate verschillen. Voorbeelden hiervan zijn de suikerstructuren die het ABO bloedgroep systeem vormen en het Rhesus (Rh) D eiwit. Naast het ABO en Rh bloedgroep systeem zijn er inmiddels nog 34 andere bloedgroep systemen geïdentificeerd met hierin in totaal meer dan 340 structuren die als een bloedgroep antigen zijn benoemd. Door het polymorfisme van deze membraanstructuren kan het ontvangen van rode bloedcellen met daarop voor de ontvanger onbekende antigenen een activatie van het immuunsysteem induceren met als resultaat de vorming van antistoffen gericht tegen deze onbekende antigenen. Bij een herexpositie aan het onbekende antigeen door bijvoorbeeld transfusie of zwangerschap zal het immuunsysteem door haar geheugencapaciteit in korte tijd gestimuleerd worden tot productie van hoge titers antistoffen met als gevolg dat de getransfundeerde bloedcellen intravasaal en/of extravasaal afgebroken worden. Een dergelijke acute hemolytische transfusiereactie kan zeer ernstig verlopen met zelfs dodelijke afloop. Het is daarom standaard praktijk om ABO en RhD compatibel bloed te transfunderen, gezien antistoffen tegen met name deze antigenen gemakkelijk zeer ernstige reacties kunnen induceren. Patiënten met een hoog risico op het vormen van antistoffen of voor wie de gevolgen van alloimmunisatie desastreus kunnen zijn ontvangen daarnaast ook bloed dat compatibel is voor andere Rh antigenen en voor het K antigen. Ondanks het effect van deze preventieve maatregelen vormen toch nog vele patiënten rode bloedcel antistoffen.

Dit proefschrift beschrijft studies naar de determinanten van rode bloedcel alloimmunisatie. Identificatie van deze determinanten stelt ons in staat om voor de individuele patiënt voorafgaand aan de bloedtransfusie een inschatting van het risico op antistofvorming te maken. Daarmee kan het huidige meer gegeneraliseerde matchingsbeleid omgebogen worden naar een patiëntspecifieke strategie met naar verwachting een reductie van antistofvorming en haar klinische en logistieke gevolgen.

Hoofdstuk 1 geeft een algemene inleiding van dit proefschrift. Het beschrijft de huidige kennis en stand van zaken met betrekking tot de pathogenese van rode bloedcel alloimmunisatie, haar klinische consequenties, methoden ter diagnostiek en huidige maatregelen ter preventie. Ten aanzien van de pathogenese spelen zowel het aangeboren als het adaptieve immuunsysteem een essentiële rol waarbij dendritische cellen in de milt een belangrijke link tussen beide systemen vormen. Andere factoren van invloed op rode bloedcel alloimmunisatie die achtereenvolgens toegelicht worden zijn: 1. de kans op blootstelling aan een onbekend rode bloedcel antigen; 2. de potentie van dit rode bloedcel antigen om het immuunsysteem te stimuleren tot antistof productie ('antigen immunogeniciteit'); 3. de genetische constitutie van de patiënt waarbij voor onder andere

diverse polymorfismen in HLA klasse II een associatie met antistof vorming na rode bloedcel transfusies is gevonden; 4. klinische condities als hemoglobinopathieën die vaak gepaard gaan met een hoge mate van antigen dispariteit tussen donor en ontvanger en daarnaast gekenmerkt worden door een chronische inflammatie.

In **hoofdstuk 2** illustreren wij dat bijna 8% van de veelvuldig getransfundeerde patiënten alloantistoffen vormen, met name gericht tegen het K, E, Jk^a en c antigen. Uitbreiding van het standaard ABO/RhD matchingsbeleid gericht op deze antigenen zou 74% van alle alloimmunisaties voorkomen, maar brengt uiteraard ook aanzienlijke kosten en logistieke beperkingen met zich mee. Voorwaarden voor antistofvorming betreffen blootstelling aan een onbekend antigen als ook een zekere mate van immunogeniciteit van dit antigen. In het verleden is deze antigen immunogeniciteit in meerdere studies geschat met de zogenaamde 'Giblett rekenmethode'. Wij bespreken diverse kanttekeningen van deze op *prevalentie*cijfers gebaseerde methode en leveren een alternatieve rekenmethode. Deze schat de immunogeniciteit van rode bloedcel antigenen op basis van *incidentie*cijfers uitgezet tegen geobjectiveerde blootstelling van het voor de ontvanger onbekende antigen. Hierbij tonen wij aan dat K het meest potente antigen is, gevolgd door E, C^w, e, Jk^a en c. Het antigen Fy^a blijkt in een niet-Kaukasische bevolking weinig immunogeen. Met name de bevindingen rondom anti-Jk^a vorming zijn opvallend en, mede ook gezien haar complementbindende vermogen, van belang voor de huidige praktijk waarin Jk^a matching tot heden nog geen hoge prioriteit verdiende.

In **hoofdstuk 3 tot 6** bespreken wij achtereenvolgens diverse klinische condities die van invloed zijn gebleken op het vormen van rode bloedcel antistoffen.

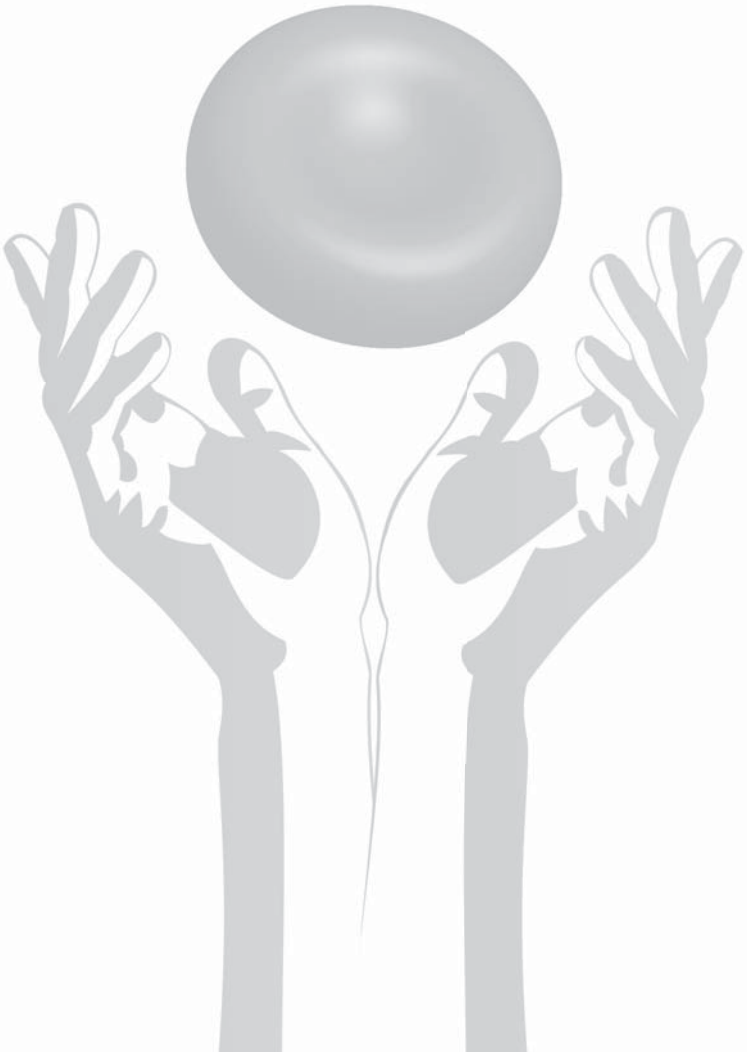
Muizenexperimenten hebben bij herhaling laten zien dat een transfusie welke ontvangen wordt tijdens een door een synthetisch viraal peptide geïnduceerde inflammatoire conditie leidt tot een versterkte alloimmunisatie respons. In analogie hieraan tonen wij in **hoofdstuk 3** aan dat rode bloedcel alloimmunisatie gemoduleerd wordt door infectieuze condities, namelijk het type infectie, de intensiteit van deze infectie en de inflammatoire respons van de patiënt op deze infectie. Wij vonden een verhoogde incidentie van alloimmunisatie in patiënten die ten tijde van ernstige bacteriële en virale infecties rode bloedcel transfusies ontvingen. Opvallend, maar ook nu in lijn met eerdere bevindingen in muizenexperimenten, vertoonden juist patiënten met Gram-negatieve bacteremiën een tweevoudige reductie van alloimmunisatie incidentie. Het lijkt dus aannemelijk te concluderen dat een specifieke inflammatoire stimulus leidt tot een specifieke immunologische uitkomst. Wij hypothetiseren een belangrijke rol voor de diverse 'Toll-Like Receptoren' die elk een specifieke intracellulaire signaalcascade kunnen aanzetten en daarmee kunnen leiden tot een uniek gen expressie profiel. Daarmee is het zelfs niet ondenkbaar dat infecties met micro-organismen van verschillende species een verschillende uitwerking op rode bloedcel alloimmunisatie hebben. Toekomstige grootschalige epidemiologische studies zullen hopelijk dit vraagstuk beantwoorden en daarmee een matchingstrategie uitgaande van een uniek klinisch inflammatoir fenotype van de patiënt doen optimaliseren.

Hoofdstuk 4 bespreekt de rol van de milt, een orgaan van belang voor B cel maturatie en ontwikkeling van IgM 'memory B cellen'. Mede gezien de milt de verbinding vormt tussen de bloed- en lymfecirculatie, is deze essentieel gebleken in de vorming van antistoffen tegen autologe en allogene hematopoëtische celantigen. Onze studie toont aan dat rode bloedcel alloimmunisatie zeer onwaarschijnlijk (maar niet uitgesloten) is na het ondergaan van een chirurgische splenectomie. Slechts één patiënt binnen een (geschat) cohort van 443 patiënten (0.23%) vormde alloantistoffen tegen rode bloedcel antigenen na een splenectomie, overeenkomend met een 20-voudig verlaagd risico. Na een splenectomie lijkt additioneel antigen matching dus niet van meerwaarde te zijn voor de Kaukasische patiënt. Alhoewel deze resultaten aansluiten bij de kennis omtrent de immunologische functie van de milt en eerder vergelijkbare conclusies zijn getrokken na splenectomie experimenten met muizen, hebben enkele retrospectieve, observationele studies in hemoglobinopathie patiënten juist een verhoogd risico op alloimmunisatie na splenectomie gesuggereerd. Andere studies vonden juist geen associatie. Wij bespreken mogelijke verklaringen voor de discrepantie van deze studies met onze bevindingen en hypothetiseren zelfs dat ook de hemoglobinopathie patiënt na chirurgische splenectomie dan wel met een functionele asplenie een gereduceerd (relatief) risico heeft op (additionele) antistof vorming. Omdat juist voor deze patiëntenpopulatie alloimmunisatie een ernstige bedreiging vormt van een optimale (transfusie)behandeling, is het van groot belang om onze bevindingen en conclusies te verifiëren in deze patiëntenpopulatie.

Als laatste illustreren wij in **hoofdstuk 5 en 6** de sterk beschermende rol van gebruik van immunosuppressieve middelen op rode bloedcel antistof vorming. In hoofdstuk 5 wordt dit besproken voor de algemeen getransfundeerde patiëntenpopulatie. Zelfs zonder correctie voor cumulatieve dosis van therapie vonden wij een vijfvoudige reductie van het risico op alloimmunisatie bij gebruik van corticosteroïden in combinatie met andere immunosuppressieve middelen. In hoofdstuk 6 wordt het risico op alloimmunisatie geanalyseerd voor de oncologische en hemato-oncologische patiëntenpopulatie. Mede door een therapie-geïnduceerde myelosuppressie ontvangen deze patiënten tijdens hun behandeling vaak een veelheid aan bloedtransfusies. Afhankelijk van de specifieke oncologische entiteit en de intensiteit van de benodigde therapie zal een zekere mate van immuungecompromitteerdheid ontstaan. Wij tonen aan dat patiënten met acute (myeloïde dan wel lymfatische) leukemie, met een myelodysplastisch syndroom (MDS), met een matuur B of T cel lymfoom, en patiënten na een autologe dan wel allogene hematopoëtische stamceltransplantatie allen een sterk verlaagd risico op alloimmunisatie hebben. Deze risicoreductie is toe te schrijven aan het sterk immuunsuppressieve karakter van de behandeling. Patiënten met deze ziekte entiteiten die om welke reden dan ook geen behandeling ondergingen, toonden een risico dat vergelijkbaar is met de algemene getransfundeerde bevolking. Daarmee dienen wij kritisch te kijken naar het huidige matchingsbeleid van MDS patiënten. Op basis van onze resultaten adviseren wij dat zowel de (geschatte) transfusie behoefte als ook het krijgen van een immuunsuppressieve

behandeling meegenomen wordt in de afweging om een MDS patiënt meer volledig gematcht bloed te verstrekken. In zijn algemeenheid zou gesteld kunnen worden dat voor de incidenteel getransfundeerde MDS patiënt geen specifieke vereisten gelden, maar dat de intensief getransfundeerde MDS patiënt op basis van een verhoogd *cumulatief* alloimmunisatie risico volledig Rh, K compatibel bloed ontvangt *tenzij* hij op dat moment behandeld wordt met sterk immunosuppressieve middelen.

Bovenstaande R-FACT studies zijn geïnitieerd met als doel om een accuraat alloimmunisatie predictiemodel op te stellen waarmee voor elke patiënt met een uniek klinisch fenotype een welafgewogen beslissing omtrent rode bloedcel antigen matching genomen kan worden. Voor deze geïndividualiseerde aanpak is het van groot belang dat het proces van alloimmunisatie begrepen wordt. In dit proefschrift hebben wij enkele klinische determinanten van rode bloedcel alloimmunisatie geïdentificeerd die als basis kunnen dienen voor een dergelijk predictiemodel. Toekomstige studies zullen gericht moeten zijn op het blootleggen van andere factoren van invloed. Na kritische evaluatie van financiële kosten en praktische uitvoerbaarheid zowel aan de donorzijde van de keten als bij het lokale transfusielaboratorium, zullen factoren op het gebied van genetische constitutie, opvoeding en omgeving, donor gerelateerde zaken, en klinische condities hopelijk uiteindelijk geïntegreerd kunnen worden in één goed functionerend risicomodel. Hiermee zal naar verwachting alloimmunisatie en haar soms desastreuze klinische gevolgen verder teruggedrongen kunnen worden.



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CURRICULUM VITAE

LIST OF PUBLICATIONS

DANKWOORD

LIST OF ABBREVIATIONS

Curriculum Vitae

Dorothea Evers was born on the 5th of January, 1982, in Rotterdam, the Netherlands. She attended secondary school at the 'Guide de Bres' in Rotterdam. In 2000, she initiated her study Medicine at the University of Leiden. As part of her medical training, she performed a four month clinical training at the Tansen Mission Hospital at Tansen, Nepal, in 2007. She obtained her medical degree in April 2008 (*cum laude*). In January 2009, she started her clinical training in Internal Medicine at the HagaHospital, The Hague (dr. M.O. van Aken) and from 2012 onwards at the Leiden University Medical Center (prof. dr. J.W. de Fijter). During her training, she participated in several committees focusing on the educational programme and on local clinical and organizational processes. From January 2013 onwards, she combined her differentiation into hematology (prof. dr. J.H.F. Falkenburg) with a PhD project at the department of Immunohematology & Blood Transfusion of the LUMC and the Center of Clinical Transfusion Research of Sanquin (prof. dr. J.G. van der Bom and prof. J.J. Zwaginga). The results from this PhD project are described and discussed in this thesis. Since June 2016, she is employed at the Radboudumc, Nijmegen, as an internist-hematologist with a special focus at transfusion medicine.

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Caram-Deelder C, van der Bom JG, Putter H, Leyte A, van de Kerkhof D, **Evers D** et al. Age of platelet concentrates and time to the next transfusion. Transfusion 2017. Manuscript accepted.

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List of abbreviations

AIHA	autoimmune hemolytic anemia
ALL	acute lymphoblastic leukemia
AMIS	antibody-mediated immune suppression'
AML	acute myeloid leukemia
APC	antigen presenting cell
ATC index	Anatomical Therapeutic Chemical index
ATG	anti-thymocyte globulin
BCR	B cell receptor
CI	confidence interval
CLL	chronic lymphatic leukemia
CNS	coagulase-negative staphylococcus
COPD	chronic obstructive pulmonary disease
DAMP	damage-associated molecular pattern
DAT	direct antiglobin test
DC	dendritic cell
DHTR	delayed hemolytic transfusion reactions
DM	diabetes mellitus
Fy	Duffy
GFR	glomerular filtration rate
HDFN	hemolytic disease of the fetus or newborn
HLA	human leucocyte antigen
HSCT	hematopoietic stem cell transplantation
IAT	indirect antiglobulin test
IL	interleukin
IQR	interquartile range
ITP	immune thrombocytopenia
Jk	Kidd
Le	Lewis
LPS	lipopolysaccharide
Lu	Lutheran
MDS	myelodysplastic syndrome
MM	multiple myeloma
MPN	myeloproliferative neoplasm
NEP	non-exofacial polymorphic structure
PAMP	pathogen-associated molecular pattern
Poly(I:C)	Polyinosinic:polycytidylic acid
PRP	pattern recognition receptor
Rh	Rhesus
RR	relative risk
SNP	single nucleotide polymorphism
TCR	T cell receptor
T_{FH}	T follicular lymphocytes
TLR	toll-like receptor
TNF	tumor necrosis factor
TRIX	Transfusie Register Irregulaire antistoffen en X(kruis)-proeven