Clinical determinants of red cell alloimmunizatiom, 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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