

Review

From Donor to Recipient: Current Questions Relating to Humoral Alloimmunization

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Abstract: Alloimmunization is an undesirable iatrogenic effect of transfusion and transplantation. In fact, recipients can be considered as responders or not responders, in a continuum from tolerance, including organ transplantation and transfusion, to polyimmunized and refractory patients. New models and large studies have enabled a better understanding of the mechanisms that induce specific alloantibody (alloAb) generation. Here, we focus on risk factors of alloimmunization. We review the alloantibody characteristics, summarize the different leukocytes involved in their induction, and suggest some hypotheses.

Keywords: transfusion; transplantation; alloimmunization; tolerance; HLA

Abbreviations: Ab: antibody; Ag: antigen; APC: antigen presenting cell; BC: blood component; Breg: regulatory B cell; Treg: regulatory T cell; GVHD: graft versus host disease; HLA: human leukocyte Ag; HPA: human platelet Ag; MHC: major histocompatibility complex; NHFTR: non hemolytic febrile transfusion reaction; NIMA: non inherited maternal Ag; PC: platelet component; RBC: red blood cells; RBCC: red blood cell component; TRALI: transfusion related acute lung injury

1. Introduction

It is estimated that every year nearly 100,000 patients benefit from organ transplantation, 20,000 from allogeneic hematopoietic stem cell transplantation, and more than 100 million from blood transfusions (<http://www.who.int/en> and <http://www.worldmarrow.org>) worldwide. Most of these medical interventions succeed and allow patients to survive their causal disease, but some recipients develop immunization against these foreign tissues, leading to rejection or impaired efficiency. Most of the alloimmunizations are Ab mediated. Alloimmunizations can occur in three ways: red blood cell component (RBCC) transfusion, in which alloimmunization rarely occurs despite the absence of an immunosuppressive regimen and frequent mismatching; organ transplantations, such as kidney grafts, in which patients can already be chronically alloimmunized against the ubiquitous foreign HLA Class I and/or the more restricted HLA Class II Ags.

Alloimmunization presents similarities and differences with infectious immunization. Both of them are influenced by Ag immunogenicity, physiology of high affinity Ab generation and inflammatory environment. However, alloimmunization occurs at a lesser rate than heterologous immunization and targets only few Ags, notably HLA Class I or II molecules (Table 1). There is a lack of reviews that look at all types of alloimmunization. In the first part, we here review the immunogenicity of the different alloAgs and the physiology of humoral post transfusional alloimmunization, and maternofetal immunization. In the second part, we focus upon Abs in transplantation, regulatory cells and the case of operational tolerant patients.

Table 1. Main alloimmunizations in clinical practice and comparison with heterologous immunization.

	Immunosuppression Regimen	Immunization Rate	Main AlloAb Specificity	HLA Expression
RBC	No	Moderate	RBC Ag RH1 and KEL1	No HLA *
		Low or very low	Other RBC Ags	
Platelets	No	Moderate	HLA Class I /II	HLA Class I *
		Very low	HPA	
Renal transplantation	No	Highly frequent	HLA Class I /II	HLA Class I/II
	Yes	Moderate		
Heterologous immunization	No	Nearly 100%	Large	No

* note that transfusion products are contaminated by Class I/II expressing WBC.

2. The Immunogenicity of Foreign Ags

Three ways of immunization can lead to alloAb generation: transfusion (RBC or PC), transplantation and pregnancy. These three pathways lead to subtle differences in alloimmunization, which is poorly explored and explained. The targeted HPA specificities are different if the immunization originates from pregnancy or PC transfusion and Ab subclasses repartition varies. After a transfusion, alloAg may be captured by dendritic cells and presented to B cells (native form) and T cells (peptide fragments bound by HLA Class II). If inappropriate co-signaling occurs, high affinity Ab-producing plasma cells will be generated in the germinal center of secondary lymphoid organs. In

pregnancy, the same mechanism will produce these cells, notably if fetal cells enter into the maternal circulation. Transplantation is more complex, even if indirect recognition shares some similarities with transfusion alloimmunization.

Transfusion, especially red blood cell (RBC) transfusion (80% of the transfusion activity), is possible because, more than a century ago, Landsteiner discovered that there were, three major groups of blood, allowing the matching of blood without creating accidents (the ABO Ags). Several decades later, another blood group was acknowledged as being strongly immunizing, the Rhesus Group. The major Ag of this group, the RH1 Ag, is assumed to be the most immunizing RBC-restricted Ag, as it easily induces immunization. Because an immunization of more than 50% is not acceptable, measures to prevent exposure of patients negative for this Ag are taken whenever possible. Interestingly, studies have shown a high variability in the rate of immunization against RH1, suggesting the involvement of other factors [1,2]. A series of observations of transfused patients revealed the potential of immunization being associated with other RBC Ags; about 20 polymorphic molecules have the capacity of immunizing a significant number of recipients (but always less than 10%, which is the case for KEL1, RH3, RH4, JK1 and FY1, for others the rate is lower than 1% [3,4]), while the remaining 300 are have not been included in the statistics. Beside RBCC transfusion, platelet component (PC) transfusion can induce immunization. PCs account for about 10% of the blood components (BCs) transfused. However, in our experience, PC transfusion is responsible for nearly half of the reported transfusion hazards. Furthermore, platelet Ags can immunize recipients, and two major types of platelet Ags must be considered. First, platelets display HLA Class I Ags, which are probably partly adsorbed from the soluble HLA molecules and partly inherited from the megakaryocytes [4,5]. Importantly, platelet HLA Class I Ags are less immunogenic than their counterparts expressed on leukocytes [6]. Second, platelets express human platelet Ags (HPA system), which are involved in achieving adhesion of platelets to the vessel epithelial wall and in hemostasis; their range of variability is much more restricted than that of the HLA system, and their immunogenicity is less frequent [7].

Multi-transfused immunocompetent recipients, such as people suffering from platelet diseases, can develop immunization in both these systems: for example, 25%–50% of Glanzmann's thrombasthenia patients develop anti-HLA and/or anti-HPA Abs [8,9], sometimes evolving to refractory states. It is clear that Ag immunogenicity is variable, depending on the recipient's HLA system, probably through its specific peptide presenting ability.

Some groups have identified HLA alleles association with susceptibility to immunization towards defined Ags. For example, HLA DRB1*04 and DRB1*15 are associated with immunization to the FY1 Ag [10,11], HLA-DRB1*01 and DRB1*10 are associated with immunization to the JK1 Ag [12], and DRB1*11 and DRB1*13 are associated with immunization to the KEL1 Ag [13]. Therefore, antigen immunogenicity varies depending on the receiver HLA typing, as some HLA molecules are better fitted to present an immunogenic peptide than others, while RH1 is probably more immunogenic, because it is a truncated protein, so numerous peptides can be presented by the recipient immune system.

Finally, the case of anti-HPA1a alloantibodies during pregnancy illustrates this concept, because HPA1 is a single amino-acid substitution and HLA-restricted. HPA exhibits two alleles: HPA1a and HPA1b. Nearly 10% of HPA1b/b mothers have been reported to develop anti-HPA1a Abs, and 85% of anti-HPA Abs are anti-HPA1a. These Abs can induce neonatal alloimmune thrombocytopenia, which occurs in nearly one of 1,250 pregnancies [14], with a risk of intracranial hemorrhage of the

fetus/newborn (in 7%–26% of the cases). The mother's HLA typing seems to have a decisive influence on the immunization risk to HPA1a: ninety percent anti-HPA1a immunized mothers carry HLA-DRB3*01:01, while only 30% of the mothers carrying both HPA1b/b (thus can develop anti-HPA1a) and HLA-DRB3*01:01 develop anti-HPA1a Abs indicating that other factors are involved. Thus, HLA-DRB3*01:01 seems to be necessary but not sufficient for inducing anti-HPA1a immunization. Recipient HLA restriction of donor HLA Class I molecules is hardly difficult, as it is highly polymorphic (both HLA and peptide fragment).

In connection with these anti-HLA Abs, some laboratories have started to consider epitopes rather than antigens, and even eplets rather than epitopes. Eplets are key polymorphic components of the donor HLA epitope. It has been demonstrated that some anti-HLA Abs bind one or two eplets of the donor HLA. One at least contains a HLA mismatch between the donor and the recipient [15].

In summary, immunogenicity depends on differences between donor and recipient (single amino acid polymorphism is less immunogenic than truncated protein), and on restriction by the HLA system of these differences (considering the fit for the mismatched peptides).

3. Alloantibodies in Transfusion

Alloimmune-induced responses to blood cell Ags are chiefly Ab-based, and more precisely IgG in nature, indicating that they result from conventional T-dependent B-cell activation and differentiation. Ab responses to alloimmunization against peptide blood cell Ags also differ from the so called “natural” Ab responses to carbohydrate moieties referred to as A, B (ABO system) and some other Ags, which originate from T-independent immune responses involving cross-reactive stimulation of extra follicular B cells by bacteria from the intestine. Low affinity IgM Abs are thus produced from iso- and not alloimmunization (these IgM Abs, however, mediate serious erythrocyte lesions responsible for massive hemolysis and cytokine storm). Natural anti-HLA Abs, probably induced by cross-reactions, are detected in some cases: some subjects share “natural” Abs against some HLA alleles without previous reported exposition. Such Abs have been poorly studied, and lab testing has demonstrated highly heterogeneous rates between patients (recipients), probably because of technical lab issues (e.g., cutoff values and methods used for detection) [16]. Some laboratories defined cutoff values on the basis of anti-HLA Abs found in healthy blood donors. The origin, significance, and clinical relevance of “natural” Abs have been poorly studied. Cross-reactions with environmental Ags are probable, and Ag denaturation in detection methods can be involved, as it exhibits some cryptic epitopes.

In the aggregated state, the model derived from most infectious agents would predict a strong stimulation and a large-scale immunization. For transfusions this is not the case, as the most immunizing epitope in transfusions is derived from the RH1 protein, which elicits a detectable immune responses in nearly 50% of cases. Unless transfusion is chronically repeated, for example in sickle cell disease patients, the frequency of detectable humoral immunization is nearly 25%, a rate much smaller than the theory would predict.

Besides the apparently stringent HLA restriction, co-signaling is known to dampen Ag presentation and APC differentiation. Because Signal 1 is probably effective, absence of inflammation—in blood products or in the recipient state—would possibly impair the alloimmune response. This raises the possibility that limited inflammation is responsible for reduced immunization, and that general genetic

predisposition modulates the susceptibility to inflammatory signals. Indeed, Signal 1 without Signal 2 leads to anergy or clonal deletion. Concerning Ab responses in terms of classes and subclasses, Ags from infectious pathogens are commonly tested for, while this is not the case for blood cell Ags.

4. Donor and Blood Product Related Factors in Transfusion

4.1. Inflammation and Alloimmunization

Additional, parallel findings indicate that in experimental models, immunogenicity of Ags can be enhanced by creating an inflammatory environment [17]. Immunogenicity is intrinsically linked to inflammation. Inflammatory factors in blood products can occur in two ways: “preparation”, which might influence proinflammatory molecule freeing during storage (*i.e.*, when there is no leukoreduction), and potentially “dangerous donors”, which might express a high rate of proinflammatory factors that stay or accumulate in blood products. Blood products indicate that inflammation is essential for immunization. In real transfusion conditions, inflammation is generally sterile, and the inflammatory response is created by several endogenous stimuli, including altered or ageing cells (RBCs, platelets), leucocytes and their residues, microparticles from different cell types, cytokines, chemokines and generally all molecules that are now ascribed as biological response modifiers (BRMs). The question of the origin of these BRMs is not clear, and there are probably multiple origins: if leucocytes are the most important providers of BRMs, contribution of each type has to be better characterized; in fact, platelets are involved in the production of BRMs, in soluble form and/or into microparticles. Finally, RBCs or long-living soluble plasmatic molecules might contribute to some detrimental effects.

It must be noted that BRMs can be infused with plasma or secreted in copious amounts by stored platelets [18]. In addition, stressed erythrocytes have been shown to present themselves with danger signals that elicit the production of proinflammatory BRMs by circulating or vessel-lining leukocytes, and by vascular endothelial cells [19]. Furthermore, it has been shown that platelets and their secreted cytokines can alter the capacity of B lymphocytes to differentiate and bias the non-specific (polyclonal) production of Ig in terms of isotypes, at least in experimental models [20]; they may also influence Ag presenting cells (such as DCs) of the recipient. The role of iron in creating inflammatory conditions in stored RBCCs is still disputed. Iron-mediated inflammatory conditions have been proven [21], and iron is released during storage, but aged (versus fresh) RBCCs do not seem to enhance alloimmunization in large-scale studies [22]. Recently it has been suggested that the collection and preparation mode of RBCCs could affect the proinflammatory status of the RBCC or the single donor apheresis platelet component in *ex vivo* experiments. However, clinical data do not support this finding. If platelets, plasmatic molecules or RBC can generate some BRMs, the main contributors are probably residual leukocytes, notably if no leukoreduction has been performed. In this way it is interesting to note that leukocytes and platelets seem to act in a synergistic manner in anti-HLA immunization [23]. Even with a leukoreduction, this immunization occurs essentially against Class I (expressed by platelets and leukocytes) and Class II (not expressed by platelets) HLA. Inversely, this synergy is not observed in RBC transfusion [24].

4.2. Residual Leukocytes and Alloimmunization

A blood product transfused to a recipient is composed of three distinct parts: (1) the desired product(s), such as RBCs or platelets; (2) some excipients—comprising anticoagulant, residual plasma and additive solutions; (3) residual leukocytes and leukocyte debris that carry HLA and non-HLA Ags, and occasionally unexpected residual cells, e.g., RBCs in PCs, and platelets in RBCCs. Some data link the presence of residual allogeneic leukocytes (potentially inflammatory factors secreting cells) with alloimmunization against RBC or platelet Ags [25,26]. Intensive leukoreduction has been experimentally shown to favor tolerance (or at least absence of Ab formation) instead of alloimmunization [25,27]. Several groups have shown that highly purified blood cell preparations infused in mice are weakly immunogenic when leukocytes are totally absent from the suspension [28]. Interestingly, there seems to be a differential role for MHC Class II negative and positive cells (such as B lymphocytes): in one study, B lymphocytes helped minimize alloimmunization to MHC Class I Ags in mice [29], while these data have been challenged by others [30]. Discrepancies between data may stem from protocol variations and viability (and/or experimental alteration) of leukocytes, eliciting either direct or indirect Ag presentation [31].

In regard to platelets, the trial to reduce alloimmunization of platelets (TRAP) (1997) provided insightful data on HLA Class I platelet immunogenicity itself, as it was clearly shown that leukoreduction (and/or leukocyte inactivation by UV irradiation) significantly reduced alloimmunization, though it did not abrogate it [25]. The HLA Class I immunization potential of platelets is large (though less frequent than leukocytes, about 18%, compared to more than 45%, respectively [25]). On the other hand, we may consider that more than 80% of PC are non-immunizing in patients, despite the fact that in the vast majority of cases there is Ag discrepancy (mismatch) between donor and recipient regarding their respective HLA Class I phenotypes. Importantly, HLA Class I in platelets often lack β 2-microglobulin, and this incomplete HLA could be adsorbed from plasma soluble forms. During storage, platelets' HLA is released from the membrane in soluble forms, enhancing complexity, as soluble molecule freeing is suspected to be linked to the transfusion related immune modulation (TRIM) effect of platelet products, although soluble forms alone cannot induce it [32,33]. Leukocytes also mediate TRIM to dampen inflammatory responses and alloimmunization, inducing tolerance mechanisms such as clonal deletion of reactive T-cells, elicitation of regulatory T-cells, anti-idiotypic T and B-cell responses, suppression of Natural Killer cell activity, switching from Th1 to Th2 type responses, selection of non-responder type immune cells, induction of apoptosis and favoring accumulation of regulatory factors, such as soluble MHC Class I molecules [34,35]. Allogeneic transfusion transfers residual donor leukocytes including DCs with the ability to present Ags. In the case of the DCs, they share at least one HLA (Class II) molecule with the recipient's immune cells, and tolerance is induced. In contrast, fully HLA mismatched transfusions are more likely to induce alloimmunization [36]. Thus, leukocytes exert a dual function in transfused patients, and it is difficult to state whether leukoreduction is beneficial or eventually detrimental for alloimmunization induction, which is currently debated. However, leukoreduction seems beneficial for many other reasons, such as reduction of discomfort, inflammatory acute transfusion reactions, NHFTR, TRALI cases, GVHD cases and transfusion transmitted infections.

5. Recipient Related Factors in Transfusion

There is evidence that an inflammatory state in the recipient favors the occurrence of alloimmunization in mice [37–39] and possibly in humans [40], and that some recipients are responders (they respond easily to alloimmune stimulation) while others are non-responders.

5.1. Genetic Risk Factors

We have discussed the role of HLA, but HLA restriction is limited to certain alloAgs, which apparently are best fitted to take place in the HLA groove. In recipients, other factors can affect the alloimmune response. Although poorly explored (TRIM 21 and CD81 have been proposed [41,42]), non-HLA genetic factors could be involved, though not necessarily in an amino acid modification manner. CD81 has been shown to be involved in the alloimmune response against RBC Ags in sickle cell disease patients, suggesting that the immunogenicity is linked to an inherited predisposition to trigger a humoral immune response through intronic polymorphisms, modifying the expression of key costimulatory proteins.

5.2. Environmental Risk Factors

Environmental factors, such as some inflammatory states, can also influence alloimmunity. Several well-documented studies have clearly shown that experimental immunization does not occur in the absence of inflammation, and, on the contrary, have indicated that this situation favors Ag-specific tolerance, sometimes in an identified active regulatory and sometimes in an immunologically ignorant way [17,37,38,43]. It is now well accepted that inflammation is instrumental to immunization after transfusion, and, moreover, that certain transfusion conditions induce inflammation. BCs can induce inflammation, but inflammation can precede transfusion.

5.3. Recipients' Tolerance to AlloAgs

Some patients do not develop alloAbs even after repeated RBC transfusions in multi-transfused patient populations, despite high frequencies of Ag mismatch [44,45]. In mouse models of RBC immunization [46,47], CD25+ T cells have been reported to mediate active tolerance to transfusion. Exploration of the Treg and Breg compartments in sickle cell disease patients revealed that alloimmunized patients displayed low rates of Treg cells, reduced levels of IL-10 and enhanced production of IFN γ compared with more tolerant patients [48,49]. In an immune thrombocytopenia model, the absence of Treg cells favored alloimmunization against platelet donor Ags and thrombocytopenia in transfused recipients [50]. It seems that exposition to alloAgs induces both immunization with T helpers and Tregs, leading to alloimmunization or specific tolerance induction, respectively. This hypothesis is supported by non-HLA genetic risk factors and could be associated with apoptosis and anergic ways of tolerance. Ignorant way of tolerance appear to be unlikely in the case of RH1 Ag, as a large amount of Ag is transfused and numerous potential presented peptides are mismatched. Concerning other mismatches with single amino acid substitution, ignorance is possible and supported by HLA restriction. Overall, alloimmunization can be viewed as a breach of tolerance. Consistently with these remarks, autoimmunity and alloimmunity against RBC have been linked

together in some studies [44,51]. Concerning platelet transfusion, alloimmunity and autoimmunity seem to also be associated in some cases [52].

In summary, multiple immunizations render transfusion programs extremely difficult to achieve and compromise survival of exposed patients. We propose a hypothesis to explain these progressive immunizations, which are not exclusive: first, multiple BC transfusions will heighten exposure of the recipient's immune system to "unknown Ags"; second, a large amount of delivered Ag can induce newly moderately fitted HLA-peptide combinations on recipient presenting cells; third, environmental conditions, *i.e.*, inflammation, can breach previous tolerance by delivering inappropriate co-stimulation signals; finally, recipients can have a high susceptibility to alloimmune responses.

6. The Pregnancy Example

The placental immune privilege is an efficient mechanism of tolerance, preventing alloimmunization against paternal Ags inherited by the newborn. In fact, high affinity IgG generation and T cell mediated cytotoxicity need a classical Ag presentation by HLA Class I and Class II, which is not allowed by trophoblastic cells, which express HLA-G to avoid natural killer cell cytotoxicity. HLA-G is a non-classical and non-polymorphic HLA molecule, so it cannot present mismatching. Unwanted obstetrical events can nevertheless induce a breach in this barrier, exposing the mother to RBCs, WBCs or platelets from the fetus in her own circulation, initiating T-dependent B cell responses and active maternal IgG based immunization against inherited paternal Ags. In pregnancy, HLA, RBC Ags and HPA can induce alloAb formation. Although anti-HLA Abs are frequently detected, they are not (or only few) pathological, while anti-RBC Ag and anti-HPA Abs are rare, but can provoke severe fetal disorders.

Immunization against HLA increases with pregnancies, 12.1%–48.5% (depending on methods, cutoff values and interval of last pregnancy) of females having carried three children or more exhibit detectable Abs (Class I and/or II) [53] without clinical detrimental consequence. In contrast, only 1/1,000 pregnant women exhibits anti-HPA Abs, mostly pathological [14,54,55]. Fetus/newborn hemolytic disease, often due to pathological maternal anti-RH1 (in RH1 negative populations), is decreasing since treatments with therapeutic Abs have been initiated. These Abs are also anti-RH1 Abs, but as they reduce the fetal RBCs lifespan, they avoid maternal immunization. Mothers can be also actively immunized by fetal platelets. Contrary to immunization against main RBC Ags, they may develop immunization anti-HPA during the first pregnancy, because HPA Ags are expressed as soon as week 16.

An additional factor besides the RH1 or HPA mismatch and HLA restriction may be the ABO phenotype (platelets display few copies of A and B Ags), which seems to affect the outcome and the severity of thrombocytopenia of the newborn [56] while it may reduce RH1 Ag immunization by reducing the fetal RBCs lifespan [57]. Isotypes of anti-HPA or anti-RH1 specific Abs have not been clearly linked with pathogenicity [58]. Finally, we have to consider fetal tolerance against maternal Ags, which has been highlighted by NIMAs tolerance. In fact, the fetal immune system more easily tolerates the mother's HLA, even if it is not transmitted. This point will be discussed below.

7. Antibodies in Transplantation

Renal transplantation is mainly discussed in this section, because the kidney is the most frequently transplanted organ and its rejection is of important clinical interest.

7.1. Donor-Specific Abs and Rejection

Candidates for transplantation often produce Abs against HLA Ags. “Natural” Abs are infrequent and Abs mostly result from alloimmunization following multiple exposures to leukocytes or platelets (blood transfusion, pregnancy), or to previous organ transplants [59]. Pre-existing alloAbs can cause hyperacute organ rejection immediately after transplantation, leading to the destruction of the graft within a few days, if targeting donor-specific Ags (DSA). *De novo* produced DSAs (post transplantation) may appear later in the recipient (mean time from 8 months to 4 years after transplantation, but can appear in the first weeks after transplantation [60]), in this case leading to acute or chronic Ab-mediated rejection (AMR), which in general, progressively impairs grafted organ functions [61]. Considerable achievement in diagnosis has recently emerged, as the single beads technology enables the detection of low rate and early DSAs. Indeed, DSAs are frequently associated with the so-called C4d-positive rejection in renal biopsies (related to humoral-mediated rejection, as C4d is a fragment of the complement system), while C4d-positive rejection is rarely DSA negative [62]. However, for reasons that are not yet fully understood, not all DSAs cause graft dysfunction [63,64], though some of them lead to subclinical rejection. Finally, a fraction of rejections are mediated by cellular cytotoxicity. The mechanisms of donor-specific Ab toxicity mostly involve two ways. The first is complement activation by the classical pathway, which triggers inflammation, recruitment and cell damage, and subsequently C4d deposition in renal graft microcirculation. The second is the Ab-dependent cell-mediated cytotoxicity of NK cells, which stimulates their cytotoxicity abilities (via killer activation receptors (KAR)), especially because of the lack of killer inhibitory receptor (KIR) ligands on transplanted cells. The Ab titration probably has prognostic significance, but needs more standardization.

Isotypes (Ig classes) and subclasses of DSA have been explored, relative to DSA cytotoxicity and graft outcomes. It is now established that IgGs are probably the only deleterious isotype. In some cases, IgMs are detected, but they are poorly related to graft outcome [62]. IgAs are frequently detected, but a correlation with graft survival has to be established [65]. Neither IgE nor IgD have been reported. Overall, it is generally considered that pathogenicity is resumed by IgGs. DSA IgGs of different subclasses have been evaluated, without clear association of any subclass with graft survival. IgG1 and IgG3 (the two complement activating subclasses) were expected to be more involved in rejection than IgG2 and IgG4, however these non-complement activating subclasses are not associated with better prognosis, suggesting other mechanisms of rejection [61,66]. The causality of immunization (pregnancy, BCs transfusion or graft) seems to have a low impact on the subclass repartition [66], and subsequently on the pathogenicity. In general, subclass analysis is not considered clinically relevant in this matter.

Years ago, the fine analysis of specificity of anti-HLA DSAs was problematic, because target cells generally harbored multiple HLA Ags. This difficulty in testing made the detection of multiple

immunizations harder. Moreover, poly-immunized patients could be tested positively for all target cells used in the labs. The emergence of single bead technology enabled better characterization of DSA specificity, because one bead stands for one allele (apart for the polymorphic DQA, which forms a heterodimer with the polymorphic DQB1). Abs against Class I HLA-A and -B, and Class II HLA-DR and HLA-DQ are clearly associated with AMR. However, the association between graft survival and DSAs against HLA-C, HLA-DQA, HLA-DRB3/4/5 and HLA-DPB1 is less clear [67–71]. The first explanation is that these Ags were difficult to explore before single bead technology and consequently less documented. The second explanation is that certain Ags are weakly expressed and therefore elicit poor immune responses and the corresponding Abs are rarely present, with low rates (when detectable), and associated with other anti-HLA Abs. Hence, the clinical significance remains elusive.

7.2. Non-HLA Abs

The hypothesis of a non-HLA component in rejection was developed during the time of indirect recognition (except natural ABO Abs) and seems to be consistent with the idea of an intrinsic predisposition to rejection. Some recipients' polymorphisms related to the innate immune system (e.g., cytokines, toll-like receptors (TLRs) and damage-associated molecular patterns (DAMPs) are interesting candidates for explaining the inherited predisposition favoring the alloimmune response, regardless of specificity, however it is still heavily debated [72]. Moreover, previously immunized patients, grafted with HLA identical donors, show greater rates of chronic rejection [73]. However, an acquired sensitization against non-HLA polymorphisms is another possibility.

In the past few years, a regain of attention focused on non-HLA alloAbs in organ transplantation as potential targets, especially on anti-endothelial cell Abs (endothelium being the main interface between the recipient's immune system and the grafted tissue). HLA Class I and II molecules are not the only proteins with high polymorphisms in humans. A potential target is the non-HLA but MHC-related protein, MICA (major histocompatibility complex class I-related chain A), which is encoded by the *MHC* gene. Indeed, *MHC* genes comprise HLA and other proteins, like MICA, which spans a highly polymorphic protein family included in the Class I *MHC* genes, as Class I HLA. MICA can be targeted by specific alloAbs, with consequent correlation with renal graft outcome [74], however a routine exploration is still discussed. Interestingly, Ags other than MICA and HLA have been proposed [75]. In summary, the clinical relevance of Abs against non-HLA Ags has to be further demonstrated in large cohort prospective studies [62].

Auto-Abs are frequently detected after transplantation, and are more frequent in rejector than non-rejector grafted patients. One reason would be that some rejectors are prone or predisposed to elicit deleterious humoral responses. Another factor would be some influence of alloimmunity on autoimmunity in such patients, like epitope spreading or cross-reactions. Some studies have tried to determine Abs with clinical value, but Ags targeted by AMR-related auto-Abs vary and are rarely reported in the different studies [76]. Thus, the involvement of auto-Abs in graft overcome is not well established, as so far only one of them has been shown to have a pathologic role, the anti-angiotensin II receptor type I Ab, which may lead to hypertension [77].

Considering bone marrow transplantation, autoantibodies are frequently detected, due to the cooperation between donor's and recipient's lymphocytes, leading to somatic hypermutation and class

switching. Thus, recipient CD8 T cells kill donor lymphocytes, and donor CD8 T cells kill recipient lymphocytes, enhancing complexity. The relations between autoimmunity and alloimmunity often occur through HLA and TCR, in the two ways.

Finally, ABO Abs have to be mentioned for two reasons: ABO Abs are a barrier for renal transplantation, although increasingly discussed now [78]. However, donors' passenger leukocytes can induce Abs against the recipient ABO system, in some cases leading to hemolytic anemia [44,79].

8. Regulatory Immune Cells in Transplantation

Inflammatory cells such as cytotoxic T cells, helper T cells, mature dendritic cells and M1-type macrophages enhance immune responses and cooperate to induce cellular mediated cytotoxicity and humoral anti-donor Ag-specific responses. This "inflammatory network" (not reviewed here) has a regulatory counterpart, which does not seem to be sufficient to overcome inflammation in current organ transplantation. Thus, an immunosuppressive drug regimen is still necessary to avoid organ rejection. Nevertheless, regulatory leukocyte populations in transplantation have been extensively studied and described for 20 years. Two leukocyte types have been proven to be particularly involved in tolerance: the first are leukocytes that have acquired regulatory properties during development in their local microenvironment, such as natural Treg (nTreg) cells [80]; the second are naïve leukocytes that differentiate peripherally into regulatory cells, such as induced Treg (iTreg) cells [81,82]. The "regulatory network" gains complexity over time and affects both cellular and humoral immunity. New leukocyte populations/subpopulations join in progressively, in innate as well as in adaptive immunity [83].

8.1. Adaptive Immunity

Adaptive immunity relative to organ transplantation reveals great complexity: induced and natural CD4⁺ Foxp3⁺ regulatory T cells are the main regulatory populations [84,85], while less frequent regulatory T cell populations such as CD8⁺ regulatory T cells [84,85], Tr1 T cells [86], double negative T cells [87], NKT cells [88] and $\gamma\delta$ T cells [89,90] are also involved in transplantation tolerance [83]. Some of these rare populations can be induced by iTreg cells (CD8⁺ regulatory T cells and Tr1), while others appear independently (nTreg, double-negative Treg). These regulatory cells are involved in tolerance and have been detected in recipients, but are insufficient for preventing organ rejection, especially when memory T cells against donor antigens are present [91]. Interestingly, iTreg cells are induced by the graft and remain at detectable levels after rejection [92].

Besides regulatory T cell populations, there has been a more recent report on regulatory B cells (Breg). Breg populations have been detected in operational tolerant (OT) patients, as IL-10 or TGF β secreting B cells with transitional T1/T2 immunophenotype (CD24⁺CD38⁺) [93–95], and have been proposed as iTreg cell inducers. B cell-induced iTreg cells seem to have powerful suppressive activities in transplantation [96]. The specificity of Breg cells has not been extensively explored yet, as well as their Ab producing capacity, class switching, affinity maturation, or plasma cell differentiation. It is thought that mainly Breg cells are B10, a heterogeneous B cell population that secrete IL-10 in large amounts to induce their regulatory activities in a CD40/BCR dependent manner. Furthermore, alloimmune tolerance can be induced by other B cell types, which could be IL-10 non-secreting cells,

such as transitional B cells. Another one could be IgM⁺ B cell clusters that have been reported in “regulatory clusters” of tolerant rats [97].

In humans, B cell recognition consistently occurs early after the graft, despite immunosuppression, without triggering an alloimmune response and rejection [98], but the regulatory activities of these clusters needs more investigation. Finally, the CD40 pathway has been proven necessary to activate the alloimmune adaptive system, but its absence protects from rejection [99,100]. CD40 is present on B cells, and its ligand, CD154, is present on T cells (both not exclusive). The CD40/CD154 pathway is considered fundamental in Ag presentation co-signaling and tolerance, it is also an essential pathway for activation and differentiation of Breg. Anti-CD40 and anti-CD154 monoclonal Abs are currently tested as kidney rejection treatments.

8.2. Innate Immune System

As for adaptive immune cells, the heterogeneity and plasticity of innate immune cells (mostly macrophages and DCs) guide them to inflammatory or regulatory contingents. The historical principle of tolerogenic immature dendritic cell versus inflammatory mature dendritic cell populations is insufficient to resume innate immunity in alloimmune intervention. A similar sub-specialization has been described for macrophages.

Macrophages were detected a long time ago as one of the dominant infiltrating cells in rejected grafts [101]. They are related to poor graft outcome [102,103] and atherosclerosis [104]. Macrophages are plastic cells with a large range of functions that depend on their activation state from M1 or M2 [105,106]. Recently, a third type of macrophage, regulatory macrophages (Mreg), has been suggested to be split off from M2 macrophages [106]. M1 macrophages are involved in phagocytosis and inflammation, while M2 macrophages seem to be more related to tissue repair (desirable) and potential fibrosis (detrimental because it leads to arteriosclerosis); in this scheme, Mreg cells would control the immune suppression [83,107]. However, few studies about Mreg implications in organ transplantation have been reported [108,109].

DCs are crucial cells for priming TD adaptive immunity, including tolerance and rejection in transplantation. Allospecific rejection can occur in three ways, involving DCs in each case: (i) direct recognition, where donor DCs are recognized by recipient T cells (donor MHC and donor peptides), mostly in acute rejections; (ii) indirect recognition, where donor peptides (notably from donor MHC) are presented by recipient MHC on DCs, mostly in chronic and humoral rejections; (iii) semi-direct recognition, where intact donor MHC-peptides are transferred to recipient DC membranes [110–114]. All three ways can lead to TD adaptive alloimmunity or tolerance, depending on DC state of maturation: immature DCs promote tolerance, while mature DCs activate effector cells. Conventional immature DC populations seem to induce iTreg cells, and inhibit B cells, T cells and NK cells, while mature DCs enhance specific Ab production. Two other subtypes have been explored more recently, with potential immunosuppressive activity: the myeloid derived suppressor cells and the mesenchymal stromal cells. Plasmacytoid dendritic cells can also promote tolerance and induce iTreg differentiation [83,115].

In summary, numerous cellular actors have been reported to take part in tolerance, but in clinical practice, this important network seems insufficient for preventing rejection when there is no

immunosuppression. Further research is needed to understand the cellular cooperation and induction, as well as tolerance initiation and enhancement.

9. Operational Tolerance (OT) in Transplantation

Operational tolerance (OT) patients are able to tolerate a transplant despite a break in immunosuppressive therapy [116]. These are rare in kidney transplants, but are more frequent in liver grafts. Cohorts of renal grafted OT are now in progress to elucidate the tolerance mechanisms. However, the few cohorts reported so far are difficult to compare and the numbers of HLA mismatches appear heterogeneous. In addition, OT patients evolve differently after a few years: they can accommodate or support durable tolerance, or sometimes switch to chronic rejection [117,118]. Infrequently, DSAs are detectable, sometimes with high titers, but generally without activating the complement system (in biopsy staining) [116]. Interestingly, OT patients seem to be low responders to blood transfusions, reinforcing the idea of genetic predisposition to alloimmunization [118]. The physiological mechanism of tolerance in transplantation may involve regulatory T cells, pDCs and mDCs ratio, $LT\gamma\delta$, as well as some cytokine polymorphisms, especially in $TGF\beta$ and IL-10 [119,120]. However, no sensitive and specific marker has been clearly identified, and it is difficult to discriminate between causal factors and consequences. Some studies have examined leukocyte populations and mRNA in PBMCs from dozens of OT patients, and concluded that there are B cell signatures in OT patients, and possible Breg cell involvement, through IL-10 or $TGF\beta$ secretion [94,95]. Nevertheless, the absence of immunosuppressive therapy is biased by immunosuppression in the control transplant patients, and these studies show a similar pattern between OT and healthy non-grafted patients. However, control patients given an immunosuppressive treatment presented a strong alteration of the B cell compartments, particularly at the time of rejection.

Overall, it is now clear that this is not immune ignorance or non-recognition of alloimmune Ags, but an active immunoregulatory phenomenon that permits graft tolerance. Indeed, cryptic allospecific reactions are detected in stable grafts in early days without acute rejection [98], suggesting that all patients develop a specific regulatory network, though insufficient for avoiding rejection.

Non-inherited maternal Ags (NIMAs) are interesting candidates for transplantation. In fact, the fetus tolerates the maternal HLA Ags, even if they are not inherited, because this exposition occurs in a tolerogenic context (during pregnancy). However, some authors explored graft survival comparing the father's versus the mother's kidneys, and no significant differences were found. It seems that the mother does not tolerate the inherited paternal Ags, and that some passenger leukocytes located within the graft can induce some proinflammatory signals, which would counterbalance the benefit of the NIMAs [121]. Nevertheless, the NIMAs are an interesting way to raise the tolerance in the case of NIMAs from other, previously not exposed donors are available. More studies are needed to confirm these hypotheses and to prove the benefit in large studies.

10. PERSPECTIVES: Coming Soon the “Regulatory Plasma Cells” (Preg)?

Plasma cells are known to be essential Ab-secreting cells of the adaptive immune response after infection. As Breg cells are a very recently characterized population, their differentiation potential has not been explored, but it can be suggested that they may differentiate and that their differentiation state

is immunosuppressive. Why are Breg cells only considered as “immunosuppressive Ag-presenting cells”, while they express a B cell receptor? Humoral tolerance may establish an evolving pathway, similarly to its immunoreactive counterpart and the ancestrally linked T cells.

Abs in transplantation are not always considered as pathogenic, and sometimes they are suspected as protective, including DSAs [122]. Auto-Abs binding DAMPs are evoked in tolerance [123–125]. Moreover, B cells are involved in transplants and transfusion tolerance. B cells differentiate into plasma cells or memory cells. Are Breg cells able to differentiate into immunosuppressive Ab-secreting cells?

Isotype characterization cannot discriminate between tolerance and rejection, thus other candidates have to be hypothesized. Some Ab glycoforms show increased immunosuppressive Ab capacity in conjunction with the affinity of the Fc fragments with the different FcRs, notably in autoimmunity and allergy. They may explain the efficiency of intravenous immunoglobulin (IVIg) therapy, when used in treatment of autoimmune diseases and graft rejection. These glycoforms depend on the enzymatic composition of the Ab-secreting cells (*i.e.*, alpha2,6-sialyltransferase), and the main mechanism may depend on the inhibitory FcRs (and alternate receptors) affinity [126–129].

In transplantation, some gene polymorphisms related to B cells, Breg cells and Abs are suspected in OT patients [94,95,130]. It can be hypothesized that a Preg cell compartment exists and takes part in the overall regulatory network, expresses alpha2,6-sialyltransferase (and/or other similar enzymatic activity), and would favor the low rate production of inhibitory glycoforms of DSAs.

11. Concluding Remarks

Alloimmunity is a long-term concern, as bone marrow transplantation, organ transplantation, and transfusions have no alternative. New immunosuppressive treatments have made it possible to reduce inefficiency and rejection, but they still have important adverse effects. Risk factors, biological markers and donor-recipient compatibility, as well as immunosuppressive optimization are serious candidates to reduce graft failures and adverse effects.

Conflicts of Interest

The authors declare no conflict of interest.

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