REVIEW

Mechanisms underlying human genetic diversity: consequence for antigraft antibody responses

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SUMMARY

This review focuses on the emerging concept of genomewide genetic variation as basis of an alloimmune response. Chronic antibody-mediated rejection is the major cause of long-term graft loss and growing evidence supports the clinical relevance of HLA but also non-HLA related alloimmune responses. Several polymorphic gene products have been identified as minor histocompatibility antigens. The formation of donor-specific alloantibodies is driven by indirect allorecognition of donor-derived peptides representing a form of conventional T-cell response. With the availability of high-throughput sequencing and genotyping technologies, the identification of genomewide genetic variation and thus mismatches between organ donors and graft recipients has become feasible. First clinical data linking genetic polymorphism and clinical outcome have been published and larger studies are currently under way. Protein arrays have successfully been used to identify a large variety of non-HLA antibodies in kidney transplant recipients and the availability of customizable peptide arrays made screening for linear epitopes on an individual patient level feasible. This review provides a summary of the recent findings in histocompatibility matching in the field of solid organ transplantation and complements it with a clear workflow for assessing the impact of genetic differences in protein-coding genes in solid organ transplantation.

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Introduction

Despite all major advances in transplant medicine and a significant reduction in acute rejection episodes with current immunosuppressive regimen, large registry analyses indicate a lack of significant improvements in long-term graft survival and a considerable proportion of grafts will fail within a decade [1,2]. Roughly, five percent of grafts are lost annually after the first year [3].

The dominant role of the ongoing alloimmune response in the graft causing chronic inflammation and resulting in the progression of fibrosis (accumulation of extracellular matrix) has been underscored [4]. The formation of antibodies directed against polymorphic donor antigens was identified as mediator of chronic graft rejection [5–8]. The human leukocyte antigens (HLA) coded in the major histocompatibility complex (MHC) on chromosome 6 represent the most important antigens in transplantation responsible for the discrimination of self and non-self. The formation of donor-specific HLA antibodies (DSA) is driven by indirect allorecognition of donor-derived peptides representing a form of

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conventional T-cell response. Screening and testing for DSA have been implemented into clinical routine [9].

Histological criteria for ABMR have been defined including the highly specific antibody-triggered C4 complement split product deposition (C4d positive ABMR) along the peritubular capillaries [9–11]. More recently, the histological entity of a C4d-negative ABMR has been introduced based on the histopathological hallmark of glomerulitis and peritubular capillaritis. The diagnosis of ABMR is now based on the presence of DSA and the typical histological findings of ABMR with or without C4d depositions [12].

Antibodies against a transplanted organ can be generally classified into two main categories: first, alloantibodies directed against polymorphic antigens that differ between the recipient and donor (HLA, minor histocompatibility antigens (mHA)), and second, autoantibodies that recognize self-antigens. If this conceptual distinction is intuitive, it is difficult to assess in the clinic. For instance, whether antibodies directed against AT-2 receptor, vimentin, collagen. . .etc, which have been recurrently reported in the circulation of transplant recipients, are directed against polymorphic parts of these proteins (alloantibodies) or rather result from a breakdown of self-tolerance (autoantibodies) has been difficult to determine up to now. This likely explains why most published studies devoted to antibody response after transplantation rather distinguished HLA and non-HLA (i.e., mHA + auto) antibodies.

There is more to rejection than HLA

There is growing evidence for non-HLA-related alloimmune responses that are destructive to transplanted organs including reports of antibody-mediated rejection following transplantation from an HLA-identical sibling [13,14]. Over 10 years post-transplant period, 20–30% of HLA-identical sibling renal allografts are lost due to rejection [15–17]. Non-HLA immunological factors are suspected to have a strong influence especially on long-term allograft function. This has been shown by Terasaki *et al.* in an analysis of UNOS registry data, deducting that 38% of graft losses are due to non-HLA immunological factors compared to only 18% associated with HLA mismatches [18]. Opelz *et al.* reported that panel reactive antibody (PRA) level is strongly associated with long-term graft loss in kidney transplants from HLA-identical sibling donors (HLA-A, B, DR matched). The effect of PRA became apparent after the first year following kidney transplantation supporting the role of non-HLA alloimmune responses in chronic allograft rejection. The presence of incompatibilities at other loci within the HLA region (e.g., DQ, DP) could not be fully excluded. However, the likelihood that such incompatibilities existed was estimated at less than 3% [19].

The importance of non-HLA antibodies in solid organ transplantation has recently been thoroughly reviewed in Nature reviews and Frontiers of Immunology [20,21]. The following review will therefore focus on an emerging new concept that allows identifying new minor histocompatibility antigens and non-HLA alloantibodies based on interindividual genetic variation using OMICS technologies. To illustrate the concept, we will include examples from both solid organ transplantation (SOT) and hematopoietic stem cell transplantation (HSCT).

Minor histocompatibility antigens

mHA are defined as any non-MHC gene product, which is sufficiently antigenic to induce CD4+ and CD8+ T cells to trigger an immune response when transplanted into another individual with absent or altered gene expression. These peptides are most importantly derived from intracellular proteins, which show polymorphisms among related and unrelated individuals. Similar to the indirect allorecognition of HLA, minor histocompatibility antigens are presented by the recipient′s own antigen-presenting cells (APC) and can trigger an alloimmune response. mHA have been extensively studied in HSCT, but not in SOT [22]. In (HLA identical) HSCT, mHAs are associated with graft-versus-host disease (GVHD) and graft-versus-leukemia effect (GVL) [23].

Most mHA are products of nonsynonymous mutations in coding sequences based on single nucleotide polymorphism (SNP) [24]. Examples for polymorphic proteins include Y chromosomal encoded proteins (HY antigens) in gender-mismatched transplantation and autosomal mHAs like the MHC class I polypeptide-related sequence A (MICA).

Genetic variation in humans

Through the implementation of high-throughput genotyping and deep sequencing technologies, an increasing amount of large-scale exome and genome sequencing data are now available. Data from large-scale sequencing projects including the HapMap Project and 1000 Genome project have shown a large genetic diversity between individuals [25,26].

The most abundant genetic variants are single nucleotide variations (SNV). A SNV is a DNA sequence

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variation where at a specific position in a single person's genome or genomes from multiple individuals two or more different bases are observed. While the term SNV itself does not demand a specific frequency of the observed alteration in a population, the term single nucleotide polymorphism (SNP) is usually used for SNVs that are observed in more than one percent of a population [27]. With the completion of phase three of the 1000 genome project 84.7 million SNPs have been identified in humans [28]. SNPs are generally further characterized based on their location within the genome and their effect on the resulting gene product. Variants located in intronic regions of the genome are generally harder to characterize as our current understanding of the structure and function of these region is limited [29]. For SNPs in coding regions, on the other hand, it is possible to infer their consequence on the resulting protein product based on existing transcript models. Major SNP resources have adapted the use of terms from the sequence ontology [30] to allow for standardized annotation of variants. Selected terms relevant for SNPs in coding regions are provided in Table 1.

The severity of the consequences caused by SNPs clearly differs. While in the case of synonymous and missense variants still viable, protein products are produced more severe variants like an early stop gain or a frameshift variant result in deterioration of the protein product and as such in loss of function (LoF) of the protein. Based on high coverage sequencing data, each individual carries on average 9184 of these nonsynonymous variants resulting in a significant protein polymorphism between individuals on the amino acid level [31].

Loss-of-function variants

Each individual carries numerous genetic variants, which cause a LoF in protein-coding genes. A study from MacArthur *et al.* on potential LoF variants (using whole genome sequencing data from 185 human individuals from the 1000 Genome Project) identified and validated rare and common LoF variants. They estimated that a typical human genome contains about 100 genuine LoF variants with about 20 affecting both alleles [32]. There is an enrichment of mutations in "disposable" or redundant pathways compared to genes essential for functional human molecular processes. This shows unexpected redundancy in the human genome and suggests that there are numerous mutations occurring at an individual level affecting the personal genome [33].

Human knockout project

Following the identification of an increasing number of LoF variants, the term human knockout project has been coined. Over the last decade, identification of such LoF variants has granted insight into basic physiological mechanisms and led to the identification of novel potential drug targets. The ACTN3 gene for example codes for alpha-actinin 3 an actin-binding protein expressed in fast twitch muscles and is phenotypically associated with better sprinting capacities [34]. A LoF variant of CCR5 (chemokine receptor type 5) that is normally expressed on the surface of leukocytes provides resistance to HIV-1 infections [35]. Individuals with a PSCK9 (proprotein convertase subtilisin/kexin type 9) knockout show reduced LDL levels, and the protein has been identified as drug target to treat hyperlipidemia. The monoclonal antibody evolocumab directed against PCSK9 has been developed [36].

LOF variants in transplantation

Following transplantation, the immune system of an individual whose genome is homozygous for a gene deletion can recognize epitopes encoded by that gene as alloantigens when a graft with one or two functioning

Table 1. Functional consequences of single nucleotide polymorphisms.

Term	Definition	Accession
Synonymous_variant	A sequence variant where there is no resulting change to the encoded amino acid	SO:0001819
Missense_variant	A sequence variant, that changes one or more bases, resulting in a different amino acid sequence but where the length is preserved	SO:0001583
Stop_gained	A sequence variant whereby at least one base of a codon is changed, resulting in a premature stop codon, leading to a shortened polypeptide	SO:0001587
Stop_lost	A sequence variant where at least one base of the terminator codon (stop) is changed, resulting in an elongated transcript	SO:0001578
Frameshift_variant	A sequence variant which causes a disruption of the translational reading frame, because the number of nucleotides inserted or deleted is not a multiple of three	SO:0001589

alleles is transplanted. The encoded proteins are new to the recipient's immune system and will be identified as alloantigens (Fig. 1).

In kidney transplantation, the well-known phenotype of post-transplant antiglomerular basement membrane disease following kidney transplantation in patients with Alport syndrome servers as a principal proof of concept. Alport syndrome is caused by mutations in COL4A3, COL4A4, and COL4A5 genes, including gene deletions that prevent the assembly of the type IV collagen, the structural component of the glomerular basement membrane. Following kidney transplantation, up to five percent of patients develop antibodies directed against the glomerular basement membrane similar to Goodpasture's syndrome [37]. Another demonstration of the impact of this phenomenon in kidney transplantation is the fact that 1/3–1/4 patients diagnosed with congenital nephrotic syndrome due to total deletion of nephrin experienced recurrence of focal and segmental glomerulosclerosis due to the development of antinephrin antibodies [38].

In the setting of HSCT, McCarroll *et al.* analyzed common gene deletions in 1345 HLA-identical sibling donor and recipient pairs and found that risk of acute graft-versus-host diseases (aGVHD) was greater when donors and recipients were mismatched for homozygous deletion of UGT2B17. A complete gene loss of the ubiquitously expressed gene UGT2B17 (UDP-glucuronosyl transferase 2B17) gave rise to multiple mHAs and resulted in a higher risk of GVHD with an OR of 2.5 (95% CI 1.4–4.6). Both cytotoxic T cell as well as alloantibody responses against different epitopes encoded by the UGT2B17 gene have been identified [39].

Santos *et al.* validated these findings in an independent cohort of 1127 patients receiving a HSCT from an HLA-identical sibling donor. UGT2B17 mismatch was present in 69 cases (6.1%). Incidence of severe aGVHD was higher in the UGT2B17-mismatched pairs (22.7% vs 14.6%, $P = 0.098$), but did not reach statistical significance. Interestingly, the association was statistically significant when a male bone marrow donor was used. It can be argued that the use of a male donor excludes the possibility of HY antigen mismatches following the combination of a male recipient with a female donor leading to the presence of multiple (potentially immunodominant) mHAs encoded on the Y chromosome [40].

Another example for the clinical relevance of a LoF donor and recipient mismatch has been reported for the GSTT1 (glutathione S-transferase theta-1) gene locus in liver transplantation that is associated with both de novo autoimmune hepatitis in the transplant and acute rejection [41]. Deletion of the GSTT1 gene that results

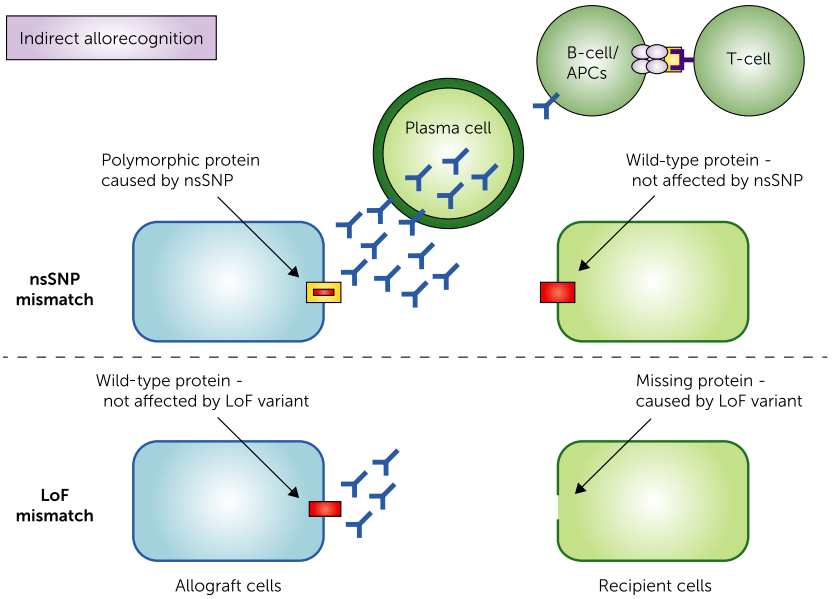

Figure 1 Concept of the indirect allorecognition of polymorphic proteins (upper panel of the image) and LoF variant mismatches (lower panel) between donors and recipients. Donor and recipient cells are depicted in blue and green, respectively. Red boxes represent a specific protein expressed by these cells, and the yellow border indicates a missense mutation. Upper panel: In case of nsSNP mismatch, the donor has a SNP giving rise to a polymorphic protein, while the recipient does not carry the same SNP. Lower panel: In case of a LoF mismatch, the donor cells express a protein that is in the recipient ablated by a LoF SNP. In both cases, the allograft introduces a new protein into the donor and the donor's immune system will start to respond to this allegedly foreign protein.

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in a complete lack of protein expression occurs in about 20% of the Caucasian population but varies among different ethnic populations (11–58%) [42]. Following liver transplantation, the development of alloantibodies directed against GSTT1 has been described. In a study by Kamei *et al.* including a total of 155 liver transplant cases, 36 patients were mismatched for GSTT1 with a functioning gene in the donor and a complete gene loss in the recipient. The incidence of acute rejection was significantly higher in these patients with an OR of 2.65 (confidence interval (CI) = 1.22–5.76, $P = 0.013$) compared to all other cases [43].

In kidney transplantation, antibodies against GSTT1 have also been described in case reports of acute or chronic antibody-mediated rejection [44].

Polymorphic proteins

Nonsynonymous SNPs that result in an altered amino acid sequence or structural changes in the protein structure add another type of potential donor versus recipient incompatibility (Fig. 1). Polymorphisms in protein-coding genes form the genetic basis of mHA. Most mHAs to date have been described in the setting of HLA-identical HSCT transplantation. Traditionally, new mHAs were identified using cell culture techniques. The antigens were then characterized from peptides eluted from the HLA molecules of alloreactive T cells using mass spectrometry [45,46].

With the development of genomewide SNP arrays, so-called whole genome association scanning (WGA) became available as efficient method for mHA discovery [47,48]. In WGA, a panel of test cells with known SNP genotypes is used to measure T-cell recognition. The list of identified autologous mHAs has since been continuously increasing and now includes over 50 proteins [49,50]. Oostvogels *et al.* recently reviewed current identification strategies for mHAs in the Journal of Bone Marrow Transplantation [51].

HY antigens

HY antigens represent another source of mHAs. Proteins on the Y chromosome show a 90% sequence similarity with their X chromosomal encoded homologs and include intracellular proteins such as RPS4Y (40S ribosomal protein S4, Y isoform 1), a ribosomal protein. The X chromosomal encoded RPS4X gene, for example, encodes a 263 amino acids protein and differs from its Y chromosomal encoded isoform by 19 amino acid substitutions based on SNPs [52].

It is well established that graft-versus-host disease (GVHD) incidence is significantly increased in male recipients receiving a HSCT from female donors [53]. Following gender-mismatched transplantation, HY antigens are recognized by the recipient's T cells as foreign antigens and can mount a combined T- and B-cell response [54,55]. Antibodies that specifically recognize recombinant HY antigens can be detected by IgG enzyme-linked immunosorbent assay (ELISA) and Western blotting. The clinical relevance of antibodies against HY antigens has been shown in HLA-identical HSCT [56]. Allogeneic antibodies against these HY antigens (including RPS4Y) that develop after HSCT are associated with an increased risk of chronic GVHD and nonrelapse [57].

In patients with acute kidney allograft rejection, the development of HY alloantibodies showed a strong correlation with acute rejection but did not correlate with C4d deposition in the biopsy or HLA antibodies [58].

On an epidemiological level, a negative impact on long-term graft function in gender-mismatched kidney transplant recipients has been shown in a retrospective cohort study in 200 000 kidney transplant recipients. Transplantation of male donor kidneys into female recipients was associated with an increased risk of graft failure during the first year (hazard ratio [HR] 1.08, 95% CI 1.03–1.14, $P = 0.003$; death censored HR 1.11, 1.04–1.19, $P = 0.003$) and between 2 and 10 years (HR 1.06, 1.01–1.10, $P = 0.008$; death censored HR 1.10, 1.05–1.16, $P < 0.001$) [59].

MICA

Major histocompatibility complex class I chain-related gene A (MICA) also represents a polymorphic peptide and about 100 alleles have been reported to date. The gene is localized in the HLA gene cluster [20]. Mismatched MICA epitopes can lead to the generation of antibodies against these MICA antigens and are associated with acute rejection episodes and reduced 1-year graft survival [60–62]. Zou *et al.* reported that antibodies against MICA alleles were detected in 217 of 1910 patients (11.4%). One-year graft-survival rate was 88.3 ± 2.2% among recipients with anti-MICA antibodies as compared with 93.0 ± 0.6% among recipients without anti-MICA antibodies ($P = 0.01$). Interestingly, the association of MICA antibodies and reduced graft survival was even more prominent in kidney transplant recipients with better HLA matching [61].

Allorecognition of different isoforms of proteins was also described in a study from Bilalic *et al.* in

presensitized dialysis patients using a different approach combining Western blotting and mass spectrometry. The group was able to show that alloantibodies are directed against specific protein isoforms, for example, vimentin 49 kDa isoform or 60 kDa isoform [63].

Autoimmunity

Several studies have reported a higher incidence of antibodies directed against protein not known to be polymorphic in patients diagnosed with chronic rejection [64]. For instance, Porcheray *et al.* observed that the majority of patients with chronic ABMR developed an antibody response to one or several of these (presumably) autoantigens at the time of rejection. Remarkably, microarray analysis showed minimal overlap between profiles, indicating that each chronic ABMR patients had developed autoantibodies to a unique set of antigenic targets [65].

This has led to the theory that chronic rejection is the result of the interplay between recipient allo- and autoimmune humoral responses [66]. Rejection of the transplant is initiated by the recognition of polymorphic donor-specific antigens (in particular the MHC molecules). Graft rejection creates an inflammatory milieu, which sets the stage for peripheral loss of tolerance. The organization of immune effector cells into functional tertiary lymphoid tissue in the graft is the site where breakdown of B-cell tolerance occurs. This intragraft microenvironment indeed interferes with peripheral deletion of autoreactive immature B cells, leading to the production of autoantibodies that will in turn accelerate graft destruction [67]. It shall however be reminded that, due to the lack of appropriate assay, the demonstration that these antibodies are really directed against a self-epitope (rather than polymorphic part of the targeted proteins) is lacking.

Anti-endothelial cell antibodies

Antibodies are massive protein (150 Kd), which limits their ability to diffuse within tissues [68], including transplanted organs [69]. As a consequence, histological lesions related to antibody-mediated rejection are mostly concentrated in the vasculature of allografts (antibody-mediated rejection used to be named vascular rejection) [70]. Therefore, from a purely practical point of view, clinicians in charge of transplanted patients might be more interested in identifying recipients with circulating antibodies able to bind to graft endothelium than the precise identification of targeted epitopes (or their nature: allo versus auto). This led to the emergence of the concept of anti-endothelial cell antibodies (AECA). Flow cytometry-based endothelial cell crossmatch tests using circulation endothelial progenitor cells and an indirect immunofluorescence test based on human umbilical cord endothelial cells have been developed. In initial studies, detection of AECA was associated with adverse transplant outcomes (higher rate of rejection and lower 1-year graft survival) [71,72]. Characterization of target antigens is still incomplete, and mechanisms of endothelial cell injury have to be resolved (e.g., complement binding, endothelial cell activation). Proteomics strategies are currently under development that should allow for a further characterization of AECA targets [73].

Association of known mHAs with outcome

In most clinical trials to date, individual mHA or a candidate list based on known mHAs has been used to test association with outcome. Dierselhuis *et al.* tested the association of a set of 15 autosomal mHAs, 10 HY antigens, and three CD31 polymorphisms and graft loss in a cohort of 444 HLA-identical sibling kidney transplantations. The incidence of graft loss was very low in this cohort ($n = 36$), and no association of mHA and graft loss could be observed [74]. It is to date the largest study to test the clinical relevance of mHAs in a HLA-identical setting in solid organ transplantation. However, an important limitation is the use of a predefined list of mHAs and the very low rate of events.

Genomics to identify new mHAs

With the availability of high-throughput sequencing and genotyping technologies, the use of genomic variations for identifying mHAs has become amenable. Computational methods leveraging publicly available SNP data to predict peptides associated with GVHD and GVL have been developed.

PeptideCheck, for instance generates peptides for all nonsynonymous SNPs stored in dbSNP (the NCBI database of genetic variation), subjects them to analysis by antigen presentation prediction algorithms and provides them together with tissue expression information and genotypic frequency data to researchers [75].

Kamei *et al.* demonstrated that mHAs recognized by a specific cytotoxic T lymphocyte (CTL) can be unraveled using genotype data. The authors grouped lymphoblastoid cell lines from HapMap based on their susceptibility to lysis by a specific CTL and performed

GWAS to identify the corresponding genetic trait in the HapMap SNP data set [47].

In their analysis of MHC class I-associated peptides (MIPs) in MHC-identical siblings, Granados *et al.* demonstrated the benefits of using subject-specific proteomic databases compiled from next generation sequencing data as reference databases for mass spectrometry (MS) analysis of MIPs. MIPs represent 8–11 amino acid long peptides that are presented in the binding groove of HLA class 1 molecules (depending on MHC restriction and immunodominance) that are potentially immunogenic. Contrary to standard proteomic databases lacking polymorphic proteins, such subject-specific proteomic databases will capture the subject's entire proteome including any polymorphic proteins. Thus, tandem MS spectra of polymorphic peptides that would remain unassigned or worse would be wrongly assigned using a standard proteomic database will be assignable to the corresponding polymorphic protein. In their analysis of two HLA-identical siblings, they found that 0.5% of all nonsynonymous SNPs between the two individuals were represented in the MIP repertoire representing mHAs [76].

First trials with genomewide approach

The first studies using a genomewide mismatch approach to identify minor histocompatibility antigens and to correlate these findings with clinical outcome have been published. Sato *et al.* performed a genomewide association study (GWAS) and tested the association of mismatched SNP alleles and the occurrence of GVHD in 1589 unrelated HLA-matched bone marrow transplant recipient pairs (10/12 HLA loci were matched, no HLA-DP matching). A total of 332 792 directly genotyped SNPs and 955 024 imputed SNPs were tested, and they were able to identify three novel mHA loci associated with grade III-IV GVHD [77].

Martin *et al.* used genomewide SNP arrays to identify 19 104 coding single nucleotide variants that predicted amino acid differences between donors and recipients in three groups of HSCT patients (HLA-identical siblings [n = 1840], unrelated donors with full HLA match [n = 194] and unrelated donors with DP mismatch [n = 1023]). In HLA-matched sibling transplants, the average mismatch in coding SNPs was 9.35% and each 1% increase was associated with an estimated 20% increase in the risk of severe grade III-IV GVHD (HR = 1.20; 95% CI = 1.05–1.37; P = 0.007) and an estimated 22% increase in the risk of stage 2–4 acute gut GVHD (HR = 1.22; 95% CI = 1.02–1.45; P = 0.03). However, no increased risk for chronic GVHD was observed. In unrelated donor and recipient pairs, the mismatch in coding SNPs was even higher and averaged 17.3%. In this setting, however, the risk for development of GVHD was mainly an effect of HLA-DP mismatch (all stages of acute GVHD and chronic GVHD) rather than genomewide mismatches and only the risk for stage 2–4 gut GVHD remained increased (HR = 1.38; 95% CI = 1.03–1.84; P = 0.03) [78].

In the setting of live donor kidney transplantation, Mesnar *et al.* performed exome sequencing in 53 donor and recipient pairs from three independent cohorts and estimated the cell surface antigen mismatch in each pair by computing the number of amino acid mismatches in transmembrane proteins. Using a mixed model, the quantitative amino acid mismatch had a significant effect on eGFR at one to 3 years after transplantation independent of HLA mismatch. Many of the polymorphisms that contributed to the mismatches had a low allele frequency, indicating that they are rare in human populations [79].

The interpretation of the study results rises questions that have to be addressed in future research efforts including MHC restriction, that is, can a polymorphic peptide be represented by the recipient's HLA molecules? Furthermore, the immunogenicity of the identified mismatched mHAs needs testing; that is, Will there be an alloimmune response? Findings have to be validated in mechanistic studies (e.g., identification of allospecific antibodies directed against predicted epitopes). Sample size is another important issue and has to be balanced to the many potential predictors of few outcomes.

High-throughput technologies to identify antigraft antibodies

Proteomics technologies can help to move from the in silico genomic level data that provide a set of individual mismatches for each donor and recipient pair to the identification of alloantibodies. Using protein and peptide arrays, it is possible to screen for antibodies against a large variety of proteins and to identify antibody reactivities to a wide array of epitopes.

Protein arrays

Minnie Sarwal's group was the first to apply proteome wide protein arrays covering up to 9000 full-length human proteins (ProtoArray, Thermo Fischer) in the transplant setting to identify non-HLA antibodies [80]. Li *et al.* correlated antibody specificities and gene expression data to identify compartment specific non-HLA targets.

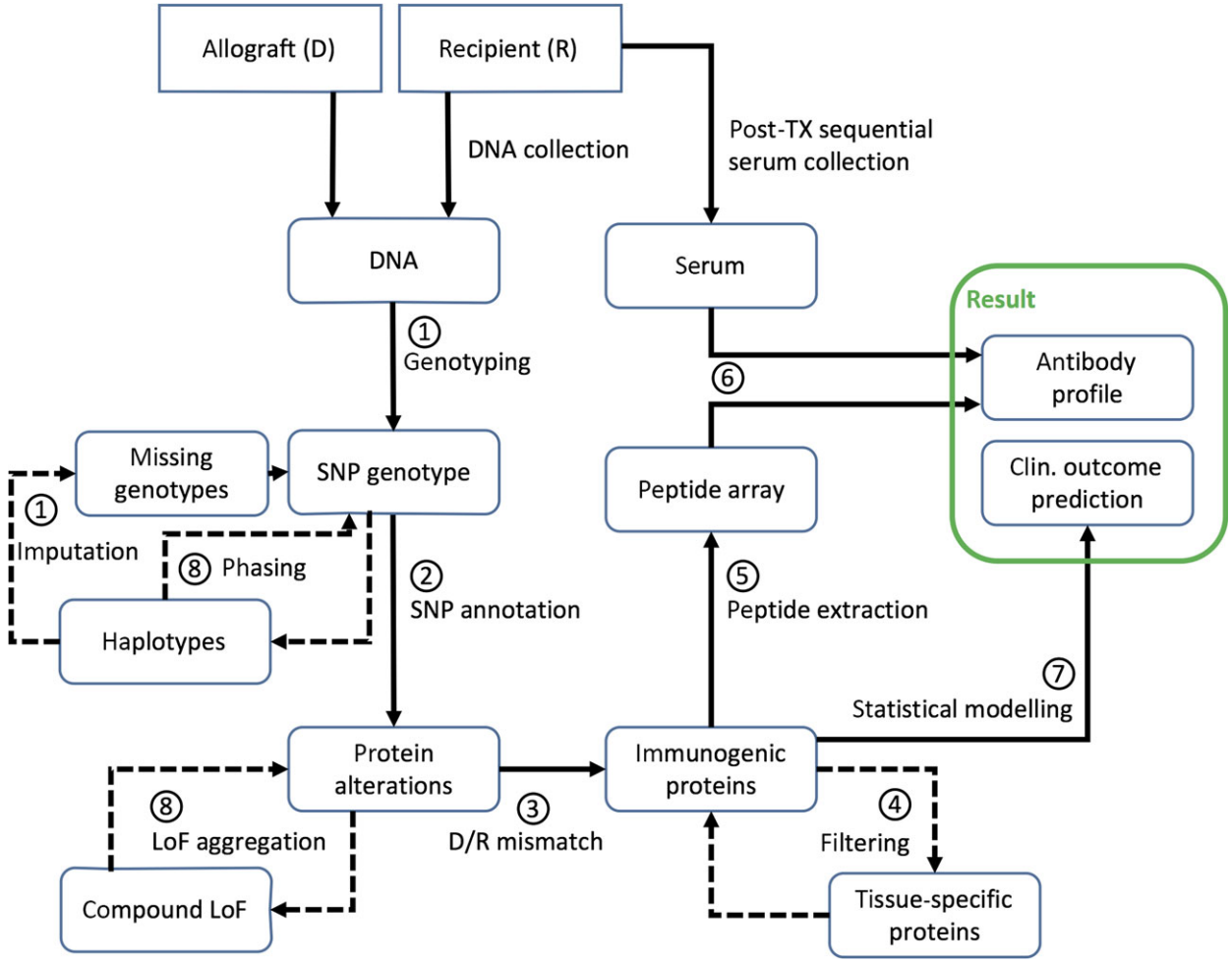

Figure 2 Workflow to identify genomewide mismatches in protein-coding genes and identification of genotype-derived donor-specific non-HLA antibodies in solid organ transplantation. (1) Donor and recipient are genotyped using either a GWAS array platform in combination with imputation or whole-exome sequencing. (2) Variant annotation is performed to predict the effect of each variant on the transcript and the protein level. (3) Mismatches between the donor and recipient resulting in polymorphic proteins that would result in allorecognition in the recipient are identified. (4) Resulting polymorphic proteins are filtered based on tissue specific expression, subcellular location, etc. to limit the analysis to immunogenic proteins expressed in the allograft. (5) Peptides representing the individual-level polymorphism are selected and prioritized based on MHC restriction and optionally intracellular or extracellular localization in transmembrane proteins. (6) Peptide arrays are spotted to cover polymorphic regions from donor-specific polymorphic proteins and complete LoF variants using 12–16 mer overlapping peptides. Longitudinal collections of serum samples from the recipient are screened for the development of donor-specific non-HLA alloantibodies directed against peptides from selected target proteins. (7) In addition, a predictor for clinical outcome based on individual immunogenic proteins or the total D/R mismatch can be derived. (8) Phasing is an optional step following genotyping, but is required to enable subsequent detection of so-called compound LoF variants that affect both alleles of a given gene that are not necessarily caused by the same SNP (e.g., stop gain at the paternal chromosome and frame shift in the chromosome inherited from the mother).

Sigdel *et al.* then showed that high-density protein arrays can be used to identify non-HLA antibodies in patients with chronic allograft nephropathy. In a cohort of 98 kidney transplant recipients, the group was able to identify 38 de novo non-HLA antibodies that were significantly associated with the development of chronic allograft nephropathy on protocol post-transplant biopsies [81]. In another study, protein arrays were used to identify target antigens of AECA isolated from the sera of recipients of kidney transplants experiencing antibody-mediated rejection in the absence of donor-specific HLA antibodies and 4 novel targets could be identified [73].

Importantly, if protein arrays do provide insight into the humoral immune responses following transplantation, polymorphisms at the amino acid level are currently not modeled by this technology (which uses a predefined set of consensus sequence-based proteins). With the identification of the epitopes targeted by

non-HLA antibodies being impossible, protein arrays do not shed light on the nature of the immune response developed by transplant recipients (allo versus auto).

Peptide arrays

Peptide arrays based on 10–14 mer overlapping peptides have been developed to circumvent the limitations of protein arrays. Peptide arrays can be adjusted to represent the amino acid substitutions that were identified on a genome level and thus may allow clarifying whether non-HLA-antibodies detected in transplant recipients are purely directed against the polymorphic parts of graft proteins (allo) or can also result from a breakdown of tolerance to self-epitopes (auto). High-density peptide arrays are now able to cover the entire human proteome in 2.1 million overlapping peptides [82]. Recently Liu *et al.* used individualized peptide arrays to screen for antibodies against linear HLA epitopes showing high sensitivity [83]. A similar approach has been applied to identify antibodies against polymorphic proteins in other diseases (e.g., multiple sclerosis) [84]. The major limitation of peptide arrays is the restriction to linear epitopes.

Future outlook

The interindividual genetic diversity in humans includes thousands of nonsynonymous SNP per individual resulting in altered protein products and a median of 20 homozygous LoF variants causing a complete gene loss in apparently healthy individuals. These variants reflect population-scale diversity as well as individual-level polymorphism. Such polymorphic proteins form the bases of mHAs. Identification of genomewide mismatches in SNV in protein-coding genes that result in altered amino acid sequences between donors and recipient provide a novel perspective to identify donor-specific mHAs. First studies in both HSCT and kidney transplantation show an association of genomewide mismatch and adverse transplant outcomes. Large cohort analysis will have to show the clinical relevance of these mismatches. For such trials, cost-effective tools for genomewide variant calling and large cohorts are required. MHC restriction of peptides as well as immunodominance and immunogenicity of the identified putative mHAs has to be addressed and add further complexity to the analysis.

High-resolution whole-exome sequencing is still expensive (roughly US $1000 per sample as of mid-2017) but provides coverage of rare variants at the individual level. Genotyping using GWAS arrays followed by imputation of uncovered variants allows the assessment of millions of SNP and may provide a cost-effective alternative (below US $100 per sample as of mid-2017) [85]. Even following imputation, however, individual-level variation will not be covered. In early 2013, the International Genetics & Translational Research in Transplantation Network (www.igenetrain.com) was founded to coordinate genetic research in transplantation across the world. The group now represents genomic and clinical data from several thousand transplant donors and recipients [86]. Studies testing the association of genomewide mismatches with both graft survival as well as long-term function in kidney transplant recipients are currently under way.

An illustration of the proposed end-to-end workflow for assessing the impact of genetic differences in protein-coding genes and to screen for new antibodies in SOT is provided in Fig. 2.

There are additional factors that contribute to the development of an alloimmune response beyond genetic polymorphism. This includes both innate and adaptive immune mechanisms (e.g., antigen presentation, immunological memory, complement regulation) as well as nonimmunological factors (e.g., adherence, ischemia–reperfusion injury) [87–91].

In summary, we discussed emerging findings and future concepts in histocompatibility matching in the field of SOT. The main goal of this endeavor is the better understanding of allo- and autoimmunity and subsequently optimal individual matching of donor organs to allograft recipients with the clear goal of prolonging transplant patency at lower maintenance immunosuppression.

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Conflicts of interest

The authors have declared no Conflicts of interest.

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