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Graft Versus Host Disease: Beyond Classical HLA Matching - A Shift in Paradigm

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Abstract

Graft-versus-host disease (GVHD), where immune response leads to injury and death of host's healthy cells, remains one of the major complications after HSCT. Hence, prevention of GVHD is an important avenue for better outcomes of HSCT patients. Most often, GVHD develops due to mismatched histocompatibility antigens between donors and recipients. Despite the full matching of HLA-A, -B, -C, -DRB1, -DQB1 loci using sequence based typing, GVHD still occurs. In this review, we focus on HLA-DP, C4 genes of MHC Class III region, and non-classical HLA genes in the development of GVHD. In addition, we discuss the role of minor histocompatibility antigens (mHA) as potential markers for occurrence of GVHD, as well as possible B cell involvement. Based on literature search and our single center HLA-DP and C4 matching studies, we postulate that HLA-DP, C4, non-classical HLA, and mHA are of potential significance in determining HSCT outcomes and can be breakthrough research area for the future. Unlike the current paradigm of T cells, mismatched HLA and mHA as the only players in the development of GVHD, the proposed shift in the paradigm is that several other factors like non-classical HLA, MHC Class III region, HLA allele-specific and non-HLA antibodies are potentially contributing towards GVHD in the presumed HLA matched recipient/donor pairs. The development and implementation of new technologies such as next generation sequencing (NGS) in clinical setting are making it feasible to have a deeper understanding of the contribution of these additional MHC loci and mHA in GVHD.

Keywords: GVHD; Classical and Non-Classical HLA; C4; mHA; NGS; Antibodies and GVHD

Abbreviations: AML-Acute Myeloid Leukemia; ALL-Acute Lymphoblastic Leukemia; APC-antigen presenting cell; CBMITR-Center for International Blood and Marrow Transplant Research; DFS-Disease Free Survival; DP-P-DP Permissive; DP-NP-DP Non-Permissive; GVHD-Graft-Versus-Host Disease; GVL-Graft Versus Leukemia; GVT-Graft Versus Tumor; HLA-Human Leukocyte Antigen; HSCT-Hematopoietic Stem Cell Transplantation; HSP-Heat Shock Proteins; HvG-Host Versus Graft; IL-1-Interleukin 1; LOH-Loss Of Heterozygosity; mHA-Minor Histocompatibility Antigens; MHC-Major Histocompatibility Complex; MRD-Matched Related Donors; MUD-Matched Unrelated Donors; NGS-Next Generation Sequencing; SBT-Sequence Based Typing; SSP-Sequence Specific Primers; SSOP-Sequence Specific Oligonucleotide Probes; TCE-T Cell Epitope; TNFα-Tumor Necrosis Factor Alpha; TRM-Transplant Related Mortality; OS-Overall Survival; UTR-Untranslated Region; WGA-Whole Genome Association

Introduction

Graft-versus -host disease (GVHD) is a major cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT), a potential curative treatment for a variety of malignant and non-malignant blood disorders refractory to chemo and radiation therapies [1-3].

Even though the two forms of GVHD (acute and chronic) have been distinguished based on the time of onset (a cutoff of

100 days), it is important to recognize that the diagnosis is based on clinical findings and other factors rather than a time frame of the occurrence of the symptoms [4,5]. The current paradigm is that GVHD manifests when transplanted donor derived T cells recognize and react to mismatched classical histocompatibility antigens (HLA) encoded by the major histocompatibility complex class I and II (MHC I and II) expressed on recipient cells [6]. Nonetheless, even with the "precise" matching of classical HLA genes, utilizing Sanger sequence-based typing (SBT), GVHD still occurs [2,6]. GVHD can significantly affect the treatment outcome and/or the quality of life of long-term survivors and occasionally can become fatal. Extensive immunosuppression to control GVHD following HSCT may lead to opportunistic infections or reactivation of certain viral, bacterial, and fungal infections, which can result in death [7-11].

While the presence of donor derived T cells with an effect on recipient's normal cells is not desirable, it is advantageous when the T cells carry a tumor-specific action against the residual or reemerging cancer cells known as graft-versus-leukemia (GVL) or graft-versus-tumor (GVT) effect. Considering that GVHD occurs in 20-60% HSCT recipients, despite full histocompatibility matching at the gene levels, an in-depth inquiry into what other factors are contributing towards GVHD following HSCT is warranted [12]. It is important to emphasize that studies reported describing the frequency of GVHD might have different definitions/criteria for histocompatibility matching. The MHC genes, coding for histocompatibility antigens, commonly called HLA in humans, are located on the short arm of human chromosome 6 [2]. The consequences of matching for HLA-A, B, C (HLA-Class I) and HLA-DR, DQ, and DP loci (HLA- Class II) vs some loci (allowing mismatches at one or two loci; mismatches at DQ, DP or mismatch at DP alone) and the level of resolution (high resolution vs low resolution) used for recipient/donor matching have been studied in depth in terms of HSCT outcome [2,3]. While some transplant programs use all classical HLA loci (HLA-A, B, C, DR, DQ and DP) for matching, others may use HLA- A, B, C, DR and DQ. It is to be noted that HLA-DP mismatches among unrelated HLA-A, B, C, DR and DQ matched recipient/donor pairs are relatively high [13]. There is emerging evidence implicating the potential roles of non-classical HLA in HSCT outcome, including GVHD. It is logical to assume that MHC class III genes (especially C4 genes) could be well involved with HSCT outcome in general and GVHD in particular because of their potential roles in controlling immune and inflammatory responses. This review examines the potential roles of the classical (A, B, C, DR, DQ, DP) and non-classical (E, F, G, H) HLA genes, the current knowledge on C4 genes of MHC Class III, a region located between MHC Class I and Class II involved in regulating immune response, and the role of mHA in GVHD, in light of the development and implementation of next generation sequencing (NGS).

Although, T cells are the most common immune cells involved in GVHD, the donor B cells have been implicated in chronic GVHD (cGvHD) as well. Various reports have shown the involvement and pathophysiology of B cell mediated cGVHD [14-16]. This review will cover the implications of the potential B cell involvement in cGVHD in selecting donors for HSCT. It is now emerging that antibodies directed against non-HLA antigens also could be potential players in GVHD after HSCT [17]. Hence, understanding the full potential of donor-derived B cells in antibody mediated GVHD (against mismatched classical HLA, non-classical HLA or non-HLA) may have implications in design and development of therapeutic regimens directed against antibody mediated GVHD - a relatively recently surfaced, but potentially underestimated event.

Pathophysiology of GVHD - The Current Paradigm

The pathophysiology of GVHD entails the damage to the host tissue prior to HSCT that may be caused by chemotherapy and/or radiation included in conditioning regimens. Pre-transplant conditioning activates host tissues, which stimulate pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α), which in turn activate the recipient's antigen presenting cells (APCs). APCs present alloantigens to donor's T cells and activate them, enhancing the expression of MHC, adhesion molecules, chemokines and the expansion of CD8+ and CD4+ T cells. Finally, phagocytes and neutrophils elicit inflammation, leading to the release of inflammatory cytokines, which along with CD8 T cells trigger the tissue destruction [1].

Classical HLA Matching and GVHD

The role of classical HLA matching in GVHD has been extensively reviewed by Petersdorf (2013) [2,18]. Polymorphism of classical HLA genes represents the most important barrier among the many factors that influence the outcome of HSCT. The number of known HLA alleles is still growing and this trend will become even more pronounced with the wider use of high throughput sequencing methods in clinical laboratories that perform histocompatibility testing. According to the international ImMunoGeneTics (IMGT) database, the current number of HLA allele sequences is 25,958 [https://www.ebi.ac.uk/ipd/imgt/hla/intro.html Accessed March 2020]. The compatibility status of each recipient/donor pair depends on the level of resolution of HLA typing and loci tested. In order to establish a common language for histocompatibility terms, Tiercy (2016) defined the following levels of resolution: low resolution referring to one field typing; intermediate resolution referring to typing results that fall between low and high resolution, and high resolution referring to two, three and four fields. Two fields designate one or a set of allotypes that share the same antigen binding site formed by the α1/α2 (coded by exons 2 and 3) domains of class I alleles and by α1 and β1 (exon 2) domains of class II alleles. In other words, these types could have different 2nd field number but their peptide binding groves have the same amino acid sequences allocating them into the same

P group. Three fields designate synonymous mutations in exons with no changes in amino acid sequences. Four fields designate non-synonymous mutations in the intronic region or in the 5 prime or 3 prime untranslated regions (5' or 3' UTR) [19]. The potential impact of non-synonymous mutations in the intronic regions is not completely understood yet, therefore our current concept of complete HLA matching is far from "complete".

To minimize GVHD, patients with primary or secondary hematologic malignancies are preferably transplanted with the "best HLA-matched donors". However, as noted earlier, even with the "high resolution" HLA matching, GVHD is not uncommon. This brings up several issues. What is "complete HLA Matching"? What other genetic and epigenetic factors are potentially involved in GVHD despite "complete HLA matching"? We will examine these in the context of NGS technology.

HLA typing is performed towards various levels of resolution using molecular methods such as sequence specific primer – polymerase chain reaction (SSP)-PCR, sequence specific oligonucleotide probes (SSOP), Sanger based DNA sequencing, and recently NGS [20]. A current standard is to type the recipient/donor pairs at A, B, C, and DRB1 loci at least (NMDP guidelines; https://bethematchclinical.org/transplant-therapy-and-donor-matching/hla-typing-and-matching/; Accessed March, 2020). Whenever possible, recipient/donor pairs are also matched at DQ and DP. In many cases, due to linkage disequilibrium a DRB1 matched recipient/donor pair may also be assumed to be matched at DQ, although there could be mismatches at the DQ level regardless. Since DP mismatches are very common in unrelated HSCT, many transplants are done across DP mismatch [13].

Next Generation Sequencing (NGS) for HLA Typing and Histocompatibility Assessment

High resolution HLA typing by DNA Sanger sequencing methodology has enabled more accurate matching of recipients and donors in allogeneic HSCT ant it has been a standard typing method until the recent development of NGS. While Sanger sequencing method provides unambiguous resolution of the most common HLA types, less common and rare types are not fully resolved, giving rise to ambiguous allele combinations. Further resolution of the ambiguities requires repeat testing using additional primers which costs time and resources [20-22]. Due to these constraints, many programs may not fully resolve rare ambiguous HLA typing results. Therefore, the possibility remains that what we call a perfect match at all loci, utilizing Sanger sequencing, in actuality may not be precisely matched. To establish if such cases contribute to the occurrence of GVHD, HLA matching needs to be determined by NGS at nearly 100% resolution.

A number of studies have discussed the benefits of NGS over the current Sanger sequencing with respect to accurate and nearly unambiguous HLA typing results. The advantages of NGS-based HLA typing approaches include high throughput by massive parallelization, clonal sequencing of single molecules, and sample multiplexing and reduced costs per sample. NGS takes a clonal approach that can handle linked polymorphism in heterozygous samples, thus eliminating the need to run additional confirmatory tests to resolve cis-trans ambiguities. The NGS system also provides extended sequence information into the intronic region variations, which could potentially influence the expression of HLA genes [20-23]. Long-term studies investigating the effect of mismatches in HLA sequences coding for non-peptide binding regions as well as in the intronic and untranslated exonic regions on GVHD may lead to a better understanding of the immunogenetics and epigenetics of GVHD pathogenesis.

The Role of HLA-DP Mismatch (High vs Low Expression of DP) in GVHD

In instances when matched related donors (MRD) are not available, HSCT has to depend on grafts from matched unrelated donors (MUD). Numerous studies have examined the relationship between HLA-DP mismatch and high risk for aGVHD [3,13]. Also, there are reports of increased life threatening GVHD among recipients with HLA-DP mismatched HSCT. HLA-DP mismatching occurs for more than 80% of otherwise HLA-matched transplant recipients and unrelated donors [13]. This contributes to substantial morbidity and mortality associated with GVHD. HLA-DP is one of the most structurally complex genes, making it difficult to assess the actual magnitude of significance of large segments of introns and untranslated regions. HLA-DP expression is associated with variations in the 3' untranslated region. The rs9277534G allele is associated with high expression of HLA-DP, while the rs9277534A allele has low expression [24]. The risk of GVHD has been observed in HLA-DPB1 mismatched transplant and influenced by the HLA-DPB1 rs9277534A expression marker. Recipients with high-expression allele had a high risk of GVHD when they received HLA-DPB1–mismatched transplants from donors with the low-expression allele [25,26]. Essentially, to determine the allele and expression levels of HLA-DP, one has to have the tool to resolve the HLA-DP genotype at a resolution level in coding, non-coding and intronic regions, which only the NGS technology can provide.

Permissive and Non-Permissive DP Mismatches Determined Utilizing a DP-T Cell Epitope Algorithm (TCE): A new algorithm based on potential T cell cross reactivity has been developed to categorize DP mismatch as permissive (DP-P) and non-permissive (DP-NP). In addition, the algorithm provides information whether the mismatch is in the graft-to-host direction (GvH) or host-to-graft (HvG) direction based on T cell epitopes (TCE) [27]. This analysis requires an unambiguous two field resolution of HLA-DP typing. At this time, this can be achieved to a great extent by NGS based typing.

Using the DPB1 T-Cell Epitope Algorithm v2.0 (2015–04), we at Rush University Medical Center (RUMC), Chicago, IL, recently reported the results of a retrospective study conducted to evaluate the clinical validity of determining HLA DP-P and DP-NP mismatch in the GvH and HvG direction. The HLA-DP TCE (T cell epitope) immunogenicity or the cross reactivity of T cells allo-reactive to the HLA-DP allele is grouped into highly, moderately, and low immunogenic. In our study, a retrospective analysis of 31 patients who underwent HSCT from a related or unrelated DP mismatched donor was performed. High resolution HLA typing based on Sanger sequencing was used to determine the recipient/donor DPB1 typing. The expected outcome was determined using the TCE algorithm. The expected outcome was then compared with the clinical outcome with respect to GVHD. Out of the 31 patients studied, the algorithm classified 15 pairs to be DP-P and 16 DP-NP. In the DP-NP transplants, 9/16 (56%) patients were expected to develop aGVHD. Two patients died due to transplant related mortality (TRM) and 6 developed cGVHD. In the DP-P transplants, the expected outcome was 0% GVHD. However, 2/15 (13%) died due to TRM, and 7/13 (54%) developed aGVHD. The severity of aGVHD was less in DP-P compared to what was observed in DP-NP mismatch. Among the 31 patients, 8 (61%) relapsed. With this limited analysis, it appears that the DP Mismatch Algorithm based on TCE seems to hold true regarding DP-NP mismatch. The TRM observed in the DP-TCE based algorithm study could have had acute and chronic GVHD or other transplant related adverse reactions [28]. While the sample size in this study is not sufficient to arrive at any valid conclusions, the algorithm is certainly worth exploring. Further multi-center retrospective and prospective evaluations are warranted to determine the role of DP in GVHD based on TCE analysis. For appropriate TCE analysis, an unambiguous 2 field typing of HLA-DP would be ideal. SBT method offers high resolution to a certain extent, however due to limited coverage, we observe several ambiguities. NGS on the other hand, facilitates complete HLA sequencing with 99.0% accuracy and much less ambiguities in HLA-DP allelic combinations. Therefore, the NGS system is of great advantage for determining the permissiveness and non-permissiveness of HLA- DP mismatch in the GvH direction and hence on the potential for GVHD.

A Shift in Paradigm

The Potential Role of Non-Classical HLA Genes in GVHD

HLA-E, -F, -G, and –H are non-classical genes of MHC class I with immunomodulatory roles [2]. There are several avenues related to alloimmune responses that have not been explored in a comprehensive way to fully assess total immune-histocompatibility between the recipient/donor pairs in gearing the alloimmune response towards more GVL and less GVHD. The roles and clinical significance pertaining to these genes in transplantation have been a subject of several recent studies [29-42].

HLA-E is a non-classical HLA antigen-presenting molecule whose potential immunomodulatory role is certainly underexplored. Similar to its classical counterparts, HLA-E is constitutively expressed on immune and endothelial cells [29,30]. However, inflammatory conditions can induce its expression on various types of cells [31,32]. Unlike the classical HLA Class I, HLA-E exhibits less polymorphism and significantly lower expression levels. Currently, there are 84 alleles, 15 distinct proteins and 1 null allele identified based on the IMGT/HLA Database (https://www.ebi.ac.uk/ipd/imgt/hla/stats.html ; Accessed March 2020). Among the 84 alleles, only two alleles namely, HLA-E*01:01 and HLA-E*01:03, differing in one amino acid in the α2 heavy chain domain have been recorded predominantly worldwide. The surface expression of HLA-E*01:01 is significantly lower compared to that of HLA-E*01:03. The two alleles exhibit distinct peptide binding affinity profiles. There are also some contradictory immunomodulatory roles for HLA-E. While it is the ligand to the inhibitory heterodimeric cell receptor CD94/NKG2A, HLA-E can also take part in immune activation by binding to the activating cell CD94/NKG2C receptor expressed by NK and CD8+ cells [33].

While its immunomodulatory effects have been recognized, its role in HSCT has not been thoroughly investigated except for a few heterogeneous, single center studies performed in small cohorts of patients. It has been suggested that HLA-E might favor tumor cell escape by evading CD8+ and NK cell immunosurveillance due to its immunomodulatory effects [34]. Previous studies have shown that the HLA-E*01:03 homozygous genotype in donors and/or recipients correlated with a lower risk of aGVHD and cGVHD [30]. Ludajic et al., (2009) reported decreased risk of aGVHD when transplanted with HLA-E*01:03 homozygous donors, and decreased risk of overall cGVHD when transplanted with HLA-E*01:01, *01:03 donors. Homozygous HLA-E*01:03 donors were found to be a risk factor for relapse and TRM. The authors noted that TRM was due to infections [35]. Contradictory to these findings, several other studies demonstrated that HLA-E*01:03 homozygous genotype in donor and/or recipient correlated with lower relapse and higher DFS [36,37]. On the other hand, Fürst et al. (2012) did not find an association between HLA-E genotypes and HSCT outcome [38]. In their previous work, Tsamadou et al. (2017), studied the effect of HLA-E polymorphism among 10/10 HLA matched unrelated HSCT in a German cohort of 509 acute leukemia patients, and observed lower TRM rates accounted for better overall survival (OS) in the HLA-E mismatched cases [39]. The authors noted that the HLA-E mismatch effect was mostly pronounced in the advanced disease group. In their most recent work, Tsamadou et al. (2019) using the Center for International Blood and Marrow Transplant Research (CIBMTR) data, authors reported the outcomes in 1,840 acute leukemia patients (acute myeloid leukemia (AML) n=1379; acute lymphoblastic

leukemia ALL n=461) who received first unmanipulated (no T-cell depletion) grafts (bone marrow or peripheral blood derived stem cells) from 10/10 HLA matched unrelated donors (MUD) in completer remission (CR) between 2000 and 2015 [34]. The patients and donors were genotyped at the HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 at second field resolution. Among those with HLA-DP mismatches, those with permissive HLA-DPB1 mismatched pairs assessed by the T-cell epitope (TCE) using the online tool from the IMGT/HLA database (https://www.ebi.ac.uk/ipd/imgt/hla/dpb.html) were included. HLA-E was typed by NGS on the Illumina platform using primers that spanned exons 2 and 3 covering all known alleles. In all, 99% of patients and donors had the HLA-E*01:01 and HLA-E*01:03 genotype. The authors found that the HLA-E*01:03 homozygous genotype in both donors and recipients has an unfavorable association with DFS (p=0.0027 and HR=1.31, p=0.0017 respectively). However, transplant from the heterozygous (HLA-E*01:01/01:03) donors showed more favorable DFS compared with HLA-E*01:03/01:03 donors (p=0.0022), indicating an unfavorable impact of a donor with the HLA-E*01:03/01:03 genotype. Overall, with respect to recipient/donor HLA-E matching, the authors did not observe any significant effect on any of the clinical outcome endpoints. This is the largest study indicating an improvement in DFS and TRM following unrelated HSCT in acute leukemia with no T cell depletion by not transplanting from HLA-E*01:03 homozygous donors [33].

The NGS based typing using CareDx AlloSeq Tx17 and AlloSeq Assign software analysis (https://www.caredx.com/alloseq-tx17/ ; Accessed March 2020) provides high-resolution genotyping of classical and non-classical HLA-E, F, G, H as well as MICA and MICB genes and it will allow us to study the role they play in the HSCT outcomes. The potential of non-classical HLA and other non-HLA molecules as part of a futuristic histocompatibility algorithm should be evaluated closely and eventually assessed in multicenter studies.

It is known that HLA-E*01:01 and *01:03 alleles exhibit significantly different surface expression levels, which could be at the transcriptional level. It is possible that in addition to the levels of expression, the differential peptide-binding profiles of the two HLA-E protein isoforms, could lead to considerable functional diversity.

HLA-G, - F, -H: HLA-G inhibits cytotoxic NK and CD8+ T cells and activates regulatory T cells [40]. To date, there are 69 HLA-G alleles that code for 19 different proteins and 3 null alleles (https://www.ebi.ac.uk/ipd/imgt/hla/stats.html; Accessed March, 2020), which arise through alternative splicing [18]. HLA-G comprises of 8 exons and 7 introns. The 14bp insertion or deletion in 3'UTR as well as +3142 C/G polymorphism have been widely studied. Both influence the HLA-G expression. Increased levels of sHLA-G have been associated with less severe GVHD and better OS. Furthermore, while low levels of sHLA-G molecules have been associated with homozygous 14bp ins/ins genotype, increased levels of sHLA-G during the first year post HSCT are independent from the +3142 C/G and the 14 bp ins/del polymorphisms [38]. On the other hand, Kanga et al. (2017) reported lower incidence of GVHD in recipients with 14bp ins/ins genotype compared with those of del/del or ins/del genotype [41].

Much less is known about HLA-F and HLA-H in relation to HSCT outcome. HLA-F has 44 alleles, coding for 6 different proteins (https://www.ebi.ac.uk/ipd/imgt/hla/stats.html; Accessed March, 2020) with HLA-F*01:01 as the most predominant allele [42]. HLA-H is defined as a pseudogene due to exon 4 deletion resulting in a stop codon, which impairs the antigen presenting function [42]. To date, 25 HLA-H alleles have been identified. More studies need to be done in a larger cohort in order to determine the role of non-classical HLA genes(https://www.ebi.ac.uk/ipd/imgt/hla/stats.html; Accessed March, 2020) .

Mismatch in C4 Genes of MHC III and GVHD

The whole HLA haplotype encompasses more than 400 genes, although in traditional matching only Class I and Class II loci are considered [43]. The MHC III region containing the genes for complement factors (i.e.C4), Factor B, as well as various other immune response genes, is not considered for matching to select the best donor in MUD transplants. In this review, we postulate that the C4 genes of MHC III can be of additional significance in donor selection in HSCT. Previous studies have shown that TNF, heat shock proteins (HSP) and other immune response genes in the Class III region are contributing factors towards the development of GVHD [44-51].

Several groups analyzed variations in the C4 genes and assessed the mismatches between recipient/donor pairs for HSCT by SNP analysis. GVHD was evaluated based on HLA match/mismatch and C4 match/mismatch [52-54]. A recent study of 225 unrelated HSCT recipient/donor pairs showed that the risk of aGVHD and cGVHD was lower among HLA matched/C4 matched donors compared to recipients who had transplants from HLA matched/C4 mismatched donors. In addition, HLA mismatched/C4 matched recipients had significantly higher chances of 5-year survival than HLA mismatched/C4 mismatched recipients [54].

Askar et al., (2015) studied 25 SNP based differences between 236 HSCT recipient/donor pairs that included a variety of hematological malignancies. The patients were in early-, intermediate- or late-stages of their respective diseases. All recipients and donors were typed for HLA–A, B, C, DR, DQ, DP and MICA by SBT and/or SSOP. SSP-PCR was used for C4 SNP analysis. The authors reported that SNPs c.2918+98G, c.3316C, and c.4385C (reference sequence C4A NG_011638.1) were

associated with severe GVHD. SNP mismatch was associated with increased risk of severe GVHD in univariate analysis (HR 2.43, 95% CI 1.32-4.47, P=0.004) and was significantly associated with severe GVHD in a multivariate analysis (HR 2.54, p=0.002) where the graft source, HLA and MICA mismatches were included [52].

We conducted a small study examining SNP mismatches in the C4 region on 52 HSCT recipient/donor pairs. Overall, 10.1% of related (2/22), 100% haploidentical (4/4) and 65.4% of recipient/matched unrelated donor (MUD) pairs (17/22) were SNP C4 mismatched. This clinical outcome study showed that SNP C4 mismatch had a higher incidence of grade 2-4 aGVHD (p = 0.044) and cGVHD (p = 0.048). Multivariate regression analysis showed higher association of SNP C4 mismatch with TRM (p = 0.020) and a trend for severe aGVHD (HR 2.450, 95% CI 0.96 – 6.22; p = 0.060) after controlling for donor type. The 25 SNPs examined span both exons and introns of C4 gene. In 46.7% C4 mismatched recipient/MUD pairs and 50% of the haploidentical recipient/donor pairs SNPs were detected in the exons. Since the exons are more related to functional proteins and introns are relevant to expression of genes, this distinction of SNPs in the exons of MUD and haploidentical cases depicts a potential significance. C4 SNPs mismatch could be a potential marker for donor selection to improve HSCT outcomes [53]. However, Moyer et al., (2018) reported no influence of C4 SNPs on GVHD in their retrospective analysis of 66 adult HSCT recipients and HLA-10/10 MUD. They found no correlation between C4 SNPs mismatch with OS, relapse, aGvHD or cGvHD with and without adjusting for DP mismatch [54]. The differences observed between this study and the other studies on the influence of C4 SNP are not understood at this time. Undoubtedly, more detailed studies are needed.

Mechanistically, the effect of C4 genes on GVHD could be due to the profile of the immune responses associated with the variations in expression of these genes. While it is inherited as a block from parent to offspring and it contributes to the haplotype makeup, it could also have variations due to recombination, deletions, additions etc. during meiotic and mitotic cell divisions. The increased survival outcome noted in HLA mismatched/C4 SNP matched HSCT could be due to reduced severity of GVHD resulting from C4 SNP match and increased GVL effect due to HLA mismatch [55].

Expectantly, NGS-based SNPs analysis of the C4 could result in a better understanding of the regions' influence on HSCT in terms of GVHD and relapse. In fact, Gendzekhadze et al., (2016) utilized an NGS method to sequence 4 PCR products spanning 12.4 kb of the C4 genes. The cohort comprised of 988 HSCT recipients transplanted at City of Hope, CA, USA (2006–2012) and their corresponding 10/10 HLA matched donors. The authors found that only 40% of pairs were identical based on the 25 previously described SNPs detected by SSP-PCR. Their NGS system detected ~300 SNPs indicating that more SNPs differences between HLA matched [10/10 or 12/12] are likely to be revealed when one resorts to NGS based sequencing system for the C4 [56].

The discrepancies noted between the published findings suggest the need for further in-depth studies to determine the role of C4 SNPs in HSCT outcome and GVHD using multiple parameters involved in the HSCT process and with an adequate number of cases while considering all the confounding factors. In this context, the latest report by Mathew et al. (2020) reports that in aGVHD of the central nervous system (CNS) there is activation of microglia with significant morphological changes and increased expression of MHC II and CD80. The authors report that the RNA sequencing data showed increased up-regulation of TNF in microglia [57]. The TNF belongs to the MHC Class III region and typing this region by NGS could potentially identify genetic polymorphisms in crucial genes in that region that affect the immune response [57]. Our previous study investigated the role of polymorphisms in TNF and other cytokines categorized as high, intermediate and low producers in GVHD using SSP-PCR. While we did not find a significant relationship between TNF and development of aGVHD or cGVHD, we did find a marginal association between development of aGVHD and donor intermediate producers of transforming growth factor-beta (TGF-β) (p=0.056) [58]. Using a mouse model Mathew et al. (2020) also demonstrated that either deleting the TNF gene or pharmacologically mediating reduction in TNF production resulted in reduction of MHC II expression by microglia. This also resulted in reduction of infiltration of Th1 and Th17 T cells, and VCAM-1+ endothelial cells and improved neurocognitive activity, while retaining the GVL effects intact [57]. Therefore, while cytokine gene polymorphisms have been studied in an isolated manner, it appears that deciphering the SNPs in the MHC Class III region, which covers several immune response associated genes, are very crucial in understanding the pathogenesis of GVHD which is the final result of the orchestration of several immune associated events as described above.

NGS Application to Understand and Institute Appropriate Immunotherapeutic Regimens against Minor Histocompatibility Antigen (mHA) Mediated GVHD

Assuming a perfect HLA match, GVHD may develop due to the involvement of minor histocompatibility antigens (mHA), which are difficult to assess by the conventional assay system. The mHAs are not routinely used in any HSCT center for recipient/donor matching. As described earlier, GVHD is mostly mediated by T cells. In HLA matched cases, the T cells recognize the mHA epitopes derived from altered self, polymorphic residues derived from the Y chromosomes (in case of female to male transplant), or autosomal antigenic residues that are different between the recipient and donor presented on the recipient's APCs or any

recipient nucleated cells [59]. The intensity and extent of mHA related GVHD will depend on the distribution of various mHA and the extent of polymorphisms within the mHA.

As Spencer et al. (2010) discussed in a comprehensive review of mHA in transplantation, to fully utilize the mHA's role in transplantation one will have to engage in the "ever advancing genomics and proteomics technology platform" [60]. Previously, efforts have been made to type human mHA [61]. However, typing of limited pre-determined mHA might not be adequate to deal with the vast number of mHA derived peptides presented to the T cells. From the entire genomic nucleotide composition point of view, humans differ from each other by only 0.1%, of the entire ~ 3 million sites along the 3-billion genomic nucleotide stretch, due to SNPs, short tandem repeats (STRs) and copy number variations (CNVs) [62,63]. This is true even among monozygotic twins who were considered to be genetically identical [64,65]. Such changes in nucleotides could result from somatic mutations that could occur in utero during gestational development, genetic recombination during meiotic cell division and biased gene conversion [66,67]. Essentially the genomes are constantly evolving, and in turn there could be constant changes in mHA as well, even in HLA identical and genetically "identical" monozygotic twins. To deal with this genetic variation situation and to reduce the mHA related GVHD, testing needs to be more user friendly with faster turnaround time, greater accuracy and reduced cost. In addition, transcriptomic and/or SNP/ SNR/CNV analyses of recipient/donor pairs also could help in selecting the appropriate recipient/donor matches to reduce the severity of GVHD.

Due to advanced array techniques to measure SNPs, whole genome association scanning (WGAs) became available as an efficient method for mHA discovery. In this approach, a panel of test cells with known SNP genotypes is used to measure T-cell recognition. T-cell recognition is subsequently investigated for association with individual SNPs to identify the genomic region that encodes the mHA. Before SNP arrays became commercially available, WGAs was performed with low-resolution genetic markers, leading to identification of large genomic regions of which all genes needed were investigated for encoding the antigen. The mHa characterized by WGAs with low-resolution markers are ACC-1Y, ACC-2, LHR-1 and HEATR1. When high-resolution SNP data are used, WGAs enables direct identification of the mHA-producing SNP or identification of small genomic regions with SNP(s) that are in linkage disequilibrium with the mHA-producing SNP. The mHA identified with high-resolution SNP are listed in Table 1 [68].

Table 1: mHA identified with high-resolution SNP (Griffioen, van Bergen & Falkenburg, 2016).

ACC-1C	LB-PRCP-1D	LB-EB13-1I	LB-APOBEC3B-1K
SLC1A5	SSR1-1S	LB-BCAT2-1R	LB-GEMIN4-1V
UGT2B17/A2	LB-WNK1-1I	LB-ARHGDIB-1R	LB-ERAP1-1R
DPH1	P2RX7	LB-PDCD11-1F	ZAPHIR
LB-SON-1R	LB-NUP133-1R	LB-SWAP70-1Q	UTA2-1
LB-FUCA2-1V			

Using mass spectrometric analysis of HLA-peptide complexes, differences in self-peptide repertoire presented by the donor and recipient HLA can be determined. Such information could lead to the generation of peptide-HLA chips or fluorescent multidimensional combinatorial encoding for typing of T cells prior to transplantation. The peptide microarray chips can then be used to detect multiple peptide-specific CD4+ or CD8+ T cells populations of T cells. CD4 (+) or CD8 (+) lymphocytes can be based on their ligand specificity [69]. The fluorescent multidimensional combinatorial encoding method involves parallel detection of multiple T-cell populations in a single sample based on their peptide-MHC complex specificity [70]. This is much like the detection of multiple single antigen-specific antibodies in one serum sample utilizing solid phase assays with color coded microbead arrays on the luminex or flow cytometry platforms.

Utilization of mHA SNP in HSCT Recipients for T Cell Based Immunotherapy To Alleviate GVHD: Despite HLA identical recipient and donor (even if DQ and DP are included in the matching process), alloreactivity still occurs through donor T cell recognition of host-derived peptides from the mHA that are different between the recipient/donor pair and bound to the self-same HLA molecules on host APCs or any nucleated cells [68]. These mHA constitute wide-ranging proteins/peptides that may possess genetic polymorphisms amongst individuals. How mHA differences can bring about GVL and GVHD is depicted in Figure 1. The intensity and extent of mHA related GVHD would depend on the tissue distribution of various mHAs and the extent of polymorphisms within the mHA, as it relates to particular recipient/donor pairs. In other words, just knowing the differences between the mHA of the recipient and donor is not entirely helpful. What

is important is which of these different specific mHAs are able to eliciting the donor T cell response against the recipient's cells/tissues/organs. If these donor specific T cells are directed against the mHA that are expressed exclusively on the hematopoietic cells, more specifically on the malignant cells, and not on other normal cells of the tissues/organs, then that would mean enhanced GVL and reduced GVHD. The information gained from the mHA diversity between the recipient/donor pair can lead to selection of T cells that are specific to immune-dominant mHA peptides exclusively expressed on the recipient's hematopoietic cells. Such T cells can be infused to enhance GVL effect with minimal GVHD effect. The importance of mHA in HSCT related GVHD is extensively reviewed by Mullally and Ritz (2007) [71].

The concepts depicted in both Figures 1A and 1B can ultimately be used to attain the Holy Grail in HSCT; to reduce GVHD and enhance GVL by dwelling into the generation of T cell responses against the expanding polymorphisms in mHAs and the presentations of the peptides by specific HLA alleles. This is where the NGS as a tool becomes pertinent in terms of reliable deciphering of the HLA alleles and the mHA polymorphisms.

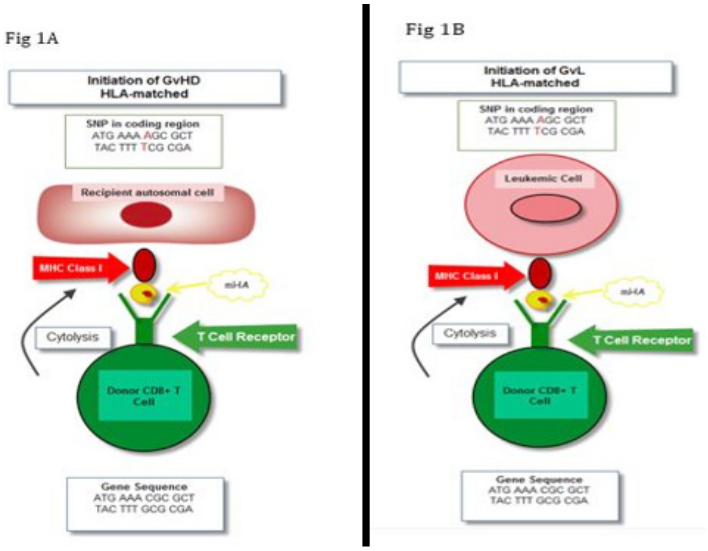

Figure 1: Initiation of GVHD and GVL.

1A. T cell recognition of mHA peptide presented by MHC class I of the recipient's autosomal cell leading to GVHD.

1B. T cell recognition of mHA peptide presented by MHC class I of leukemic cell leading to GVL.

The mHA diversity will keep increasing based on environmental exposures, tissue damage due to infections, immune responses, etc. The presentation by the appropriate HLA will be the critical factor in eliciting an immune response. Full comprehension of the mHA differences between the patient/donor pair in terms of HLA types and the potentials to develop GVHD or GVL will require more detailed investigations.

How does NGS Based HLA Typing Allow us to Refine the mHA and T Cell Response [An Extension of What We Know Already about the Genetics and Immune Responses against mHA in Eliciting or Modulating GVHD]?: Despite complete HLA matching at the allelic level [two field resolution without ambiguities facilitated by the NGS platform in a great percentage of cases] we still have to deal with GVHD and relapse. The question arises as to what exactly are the engrafted donor T cells recognizing? This could be the peptide bound to the groove formed by the α1 and α2 chains of the Class I HLA or the α1 and β1 chains of the HLA Class II molecules or the combination of the conformation created by the HLA molecule and the peptide moiety in the groove. What remains to be answered is also whether the changes in the α3 segment of the HLA Class I affect the configuration of the groove and the peptides that bind to that groove.

Lansford et al. (2018) recently reported a computational mHA prediction method that combines recipient/donor genotyping data with RNA sequencing data from reference human tissue and leukemia samples to predict mHAs with high binding affinity to HLA that are expressed in specific tissues. They used a method to predict tissue-restricted mHAs in a cohort of 101 patients who had undergone allogeneic HSCT for myeloid neoplasms and had been genotyped for 13,917 nonsynonymous coding single-nucleotide polymorphisms (cSNPs). They discovered a new leukemia-associated antigen by performing targeted mass spectrometry coupled to differential ion mobility spectrometry (DIMS-MS), followed by detection of antigen-specific T-cell populations using peptide/MHC tetramers [72]. This approach will lead to newer technology-driven T cell based immunotherapeutics following transplant utilizing the fundamental mechanistic aspects of GVHD in otherwise HLA matched recipient/donor HSCT.

In essence, knowing the exact HLA allele, which can be achieved by the NGS platform, will be required for predicting or assessing the potential immunogenicity of the mHA peptide bound to the peptide binding groove to elicit an immune response against the host by the donor's T cells.

Potential Role of B Cells in GVHD

We already know that T cells are the main drivers of GVHD. However, there is evidence to support the role of B cells and hence antibodies in both aGVHD and cGVHD. A review by Sarantopoulos et al., (2007) summarizes the studies on the role of B cells in cGVHD and also addresses the potential pathogenesis mechanisms [73]. Understanding the mechanistic roles of B cells and antibodies in the development of GVHD has helped exploration of newer and more appropriate therapeutics against GVHD. They also reviewed some of the pertinent mechanisms responsible for persistent B cell activation and loss of B cell tolerance in patients

with cGVHD, which includes recent studies in preclinical models that have identified novel B cell directed agents that may be effective for prevention or treatment of cGVHD. Studies show that about 30%-50% of patients with HLA-matched sibling donors and 50-70% of patients with unrelated donors develop cGVHD around 4 to 6 months after HSCT [74-76]. This brings up the issue of the level of HLA matching between recipients and donors and also the potential of mHAs peptides in leading towards acute and chronic rejections. Kamble et al. (2006) found that depleting B cell by rituximab (monoclonal antibodies against CD20) showed favorable effects of rituximab on resistant aGVHD outcome [77]. Other studies showed that rituximab given as part of a myeloablative or nonmyeloablative conditioning regimen or given before or after transplantation led to lower-than-expected rates of GVHD [78-83]. Studies so far indicate that rituximab administered prior to or immediately after transplantation is safe and that there are no adverse effects on the engraftment process [78,79]. However, as one should expect, the B-cell recovery is likely to be delayed. Also, it is known that apheresis products with high numbers of B cells results in higher incidence of GVHD and increased TRM [84].

Normally, the HSCT donors' HLA antibody status is considered immaterial for stem cell donation since the HSCT allografts do not contain any circulating blood although the eligible donors with multiple pregnancies or organ transplants are likely to have multiple HLA antibodies. However, recent studies are indicating that the stem cell grafts may contain allo-sensitized B cells if they are derived from previously allo-sensitized individuals which could differentiate into antibody-producing plasma cells [84]. By extrapolation, it is possible that memory B cells could be present against the mismatched HLA antigens of the recipient in the context of HSCT. These donor-derived recipient-specific HLA antibodies (DRSHA) have been shown to be involved in GVHD [85].

Hence, it is possible that such donor derived passenger B lymphocytes capable of maturing into plasma cells and destined to secrete HLA specific antibodies could have antibody mediated GVHD should the antibodies be directed against the recipient HLA in a mismatched related or unrelated transplantation and especially in haploidentical transplantation. Taniguchi et al., (2012) investigated the presence of circulating HLA antibodies in 123 related solid-phase single bead antigen assays. Of these, 6/27 (22%) parous female and 1/57 (1.8%) male, donors were HLA antibody positive. None of the non-parous donors in this study had any detectable antibodies. The authors then determined HLA antibody levels in seven patients who received HSCT from antibody positive donors. Of these, four recipients became HLA antibody positive after HSCT. The authors report that the specificities of the antibodies that were detected in these patients post-transplant closely resembled those of the antibodies found in the donors, indicating their production by donor-derived plasma cells. Apparently, the kinetics of the HLA antibody levels were similar in all four, with levels of HLA antibodies increasing within 1 week of HSCT and peaking at days 10-21, followed by steady decline. While the allotype of these HLA antibodies were not characterized, the authors claim that based on the specificities and kinetics they are most likely to be derived from the passenger B cells from the allograft [86].

Therefore, the presence of donor B cells in the stem cell graft could activate and expand the alloreactive donor T cells by cognate interaction with alloantigens. These alloreactive donor T cells could be directed against mismatched HLA or mHA. This is interesting in the sense that the B cell mediated GVHD is not necessarily due to antibodies, but due to T cells activated by the donor B cells as depicted in Figure 2. This newfound role of B cells could result in novel therapeutic targets to deal with GVHD following HSCT by targeting T cells and B cells [87-93].

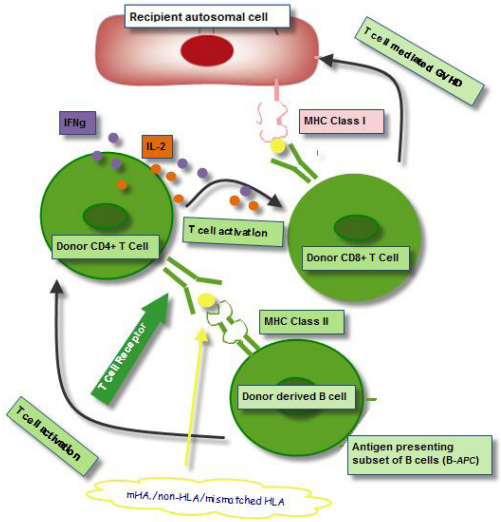

Figure 2: Antigen presenting B cells activating T cells and leading to T cell mediated GvHD.

A hypothetical situation where the donor's antigen presenting B cell subtype [B-APC] might present mismatched HLA/non-HLA/mHA to the recipient's CD4 T cells which in turn can activate the CD8 T cells of the donor to have deleterious effects on recipient's autosomal non-hematopoietic cells through cytokine mediated events [94].

Testing the Donors for HLA Antibodies and its Potential Relevance in Preventing GVHD

This affirmation of B cells' role in GVHD brings us to the issue of testing the eligible donors for HLA antibodies because of the potential presence of B cells in the graft. In the current practice of HSCT the recipients are tested by many centers for

antibodies against the mismatched HLA antigens due to the possibility of pre-existing donor-specific HLA antibodies against the mismatched HLA to avoid potential engraftment failure. From the GVHD standpoint the issue arises whether the donors with pre-existing recipient-specific antibodies (against the mismatched recipients' HLA antigens) should be excluded. This is assuming that the donor's stem cell graft could have memory B cells that are against the mismatched alloantigens which could potentially lead to B cell mediated GVHD. The screening of HSCT donors for HLA antibodies is not a common practice at most HSCT transplant centers as of now, however, this is an area that needs to be explored in terms of dealing with GVHD from a new direction. Furthermore, the B cells could be recognizing both mHA and HLA with the help of T cells and could be influenced by the genes in the MHC III region as well.

Role of NGS based HLA Typing and Current Technology for Detection of Allele-Specific HLA Antibodies in Deciphering the Specificity of HLA Antibodies Carried by the Potential Memory B cells of the Donor's Hematopoietic Cells Present in the Graft: As we appreciate the role of antibodies in GHVD, we have to realize that the antibodies can be very allele-specific even among broadly allosensitized individuals. For example, it is not unusual to have antibodies against just one allele of a parental HLA antigen only (for example only against A*02:01 and not against A*02:03 or *02:06 etc; or against DQB1*06:01 and not against DQB1*06:02 or DQB1*06:03 etc). This is depicted in Figure 3 showing a hypothetical example of HSCT recipient/donor pair HLA-A typing performed by NGS where the recipient is A*02:01, A*03:01 and the donor A*03:01, A*02:642 donor. Current antibody assays can distinguish antibodies against several known specific HLA alleles. We know that allele specific antibodies are involved in pathological reactions by recognizing and reacting towards specific HLA alleles with evidence in antibody mediated acute immunological rejections in renal and other solid organ transplants [87,88]. The GVHD in HSCT is similar to the immunological reactivity of the recipients' antibodies against the donor's HLA alleles in solid organ transplant which is essentially host vs graft disease (HVGD) in solid organ transplants. Hence when it comes to selecting acceptable donor/ recipient pairs in terms of avoiding potential/antibody mediated GVHD, the high resolution typing of the donor and the recipient is of crucial importance. With the high resolution HLA typing of the recipient and donors by the NGS platform and with the current ability to detect several allele specific antibodies, we are in a position (compared to the era prior to the introduction of NGS platform based HLA typing and allele-specific HLA antibody detection systems) to potentially assess the role of antibodies in GVHD in HSCT. This is an evolving area and we will have to wait to fully understand the role of B cells and antibodies and the CD4+ T cells which help the B cells in mediating GVHD.

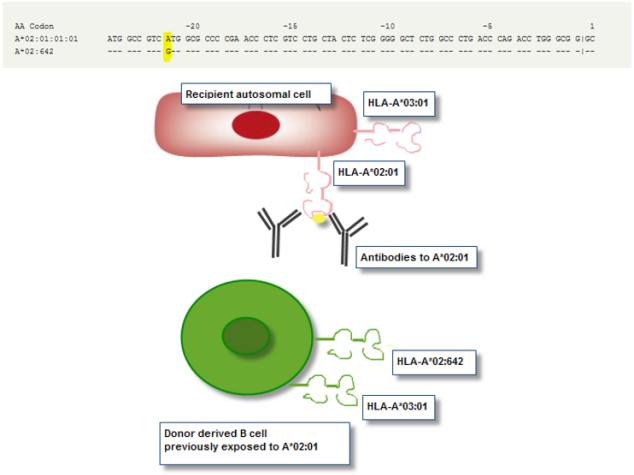

Figure 3: Antibody mediated GvHD.

Top image shows the difference in exon 1 nucleotide sequences between the A*02:01 and A*02:642. Minimum requirement for IMGT for Class I is to sequence regions exon 2 and 3 [95]. Therefore, if exon 1 was not sequenced it is likely that this typing would be reported as a P or G group i.e. A*02:01P includes ambiguous A*02:642 typing (http://hla.alleles.org/alleles/p_groups.html; Accessed March, 2020). Considering that A*02:642 is not a common and well document allele, and if exon 1 was not sequenced this ambiguity would likely not be resolved to a full extend by Sanger Sequencing. However, if the donor is truly A*02:642, was previously sensitized to A*02:01, and is making allelic A*02:01 antibody that could have a long-term effect on recipient after HSCT. Bottom image represents a hypothetical case where this occurs and it shows a potential for B cell mediated GVHD based on antibody to A*02:01.

An Important Twist in the Antibody Mediated GVHD: Potential Non-HLA Antibody Mediated GVHD: We are increasingly becoming aware of the fact that there are likely to be non-HLA related antibodies directed against the selected donor in the recipient, which could cause immunological injuries to the allograft regardless of HLA matching affecting long-term survival of solid organ allografts [17]. Therefore, by the same token, if the selected HSCT donor has memory B cells that are potentially sensitized towards certain non-HLA antigens of the recipients, the new emerging evidence suggests that such non-HLA antibodies against certain tissue specific antigens could lead to GVHD [89]. This is an interesting and evolving field in both solid organ transplant and HSCT. We are not certain about the polymorphic nature of all of the known non-HLA genes, although, we do know that some are polymorphic [90,91]. The currently known and studied non-HLA antibodies associated with immunological injuries of solid organ allografts are listed in Table 2. In addition,

tissue injuries can expose cryptic or altered self-antigens towards which the donor B/T cells could elicit an immune response regardless of full HLA matching [92]. It can be hypothesized that such altered self-antigens could also be expressed due to inflammatory conditions following transplantation.

Vimentin (VM)	Enolase 1 (ENO1)	Chromatin assembly factor 1 subunit B (CHAF1B)	Leucine-rich repeat transmembrane protein (FLRT2)
Angiotensin (AGT)	CD36	Glutathione S-transferase theta-1 (GSTT1)	Interferon-induced helicase C domain-containing protein (1FIH1)
Peroxisomal trans-2-enoyl-CoA reductase (PECR)	Myosin	Nucleolar and spindle-associated protein 1 (NUSAP1)	Aurora kinase A-interacting protein (ARKA)
CXCL11	Heterogeneous Ribonucleoprotein K (HNRNPK)		Peptidyl-prolyl cis-trans isomerase A (PPIA)
CXCL9	Tubulin alpha 1B (TUBA1B)		Eukaryotic translation initiation factor 2A (EIF2A)
Agrin (AGRN)	LG3		Prelamin-A/C (LMNA)
Interferon gamma (IFNG)			Protein kinase C zeta type (PRKCZ)
Secretory phospholipase A2 receptor (PLA2R)			Protein kinase C eta type (PRKCH)
Receptor-type tyrosine-protein phosphatase-like N (PTPRN)			Lamin-B1 (LMNB)
Regenerating islet-derived protein 3-alpha (REG3A)			CXCL10
Basement membrane-specific heparan sulfate proteoglycan core protein (LG3)			Rho GDP-dissociation inhibitor 2 (ARHGDIB)
			Glial cell line-derived neurotrophic factor (GDNF)
			Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
			Tumor necrosis factor (TNFA)

Table 2: A non-HLA targets using single non-HLA antigen coated beads on the Luminex platform adopted from One Lambda LabScreen Autoantibody product insert (https://www.onelambda.com/en/applications/autoantibody.html; Accessed March 2020).

Recently, Pirotte et al. (2018) reported the case of a GVHD involving the CNS (ingravescent encephalopathy). This was a 58 year old man who had an allo HSCT. While the conventional imaging did not show any CNS immune-mediated lesions, serum analysis showed presence of anti-neuronal antibodies directed against anti-contactin-associated protein 2 (anti-Caspr2), a protein associated with voltage-gated potassium neuronal channels. Functional imaging with 2-deoxy-2-[fluorine-18] fluoro- d-glucose integrated with computed tomography (18F-FDG PET-CT) demonstrated diffuse cortical and subcortical hypometabolism. The patient recovered with intact cognitive functions after treatment with a combination of immunosuppressants [89].

In this context, it is possible that the GVHD potentially due to mHA following autologous HSCT could be caused by the mHA generated during the conditioning regimen (chemotherapeutic or radiation treatment) previous to the autologous HSCT.

Hence, antibody mediated GVHD [cGVHD] has broader dimensions –mismatched HLA, the mHA and the non-HLA antigens that are all potential players. We do need further detailed studies to clearly understand the exact role of B cells from these perspectives in terms of pathogenesis of B cell mediated GVHD in HSCT and to further elucidate the potential targets and potential novel therapeutics.

Discussion

Sequencing of exons of various HLA loci pertaining to the peptide binding grove alone may not be sufficient to fully understand the role of HLA in histocompatibility between recipient/donor pairs in transplantation and in associating certain diseases with specific HLA genes or haplotypes. The expression of each locus varies depending on the control elements within the gene and potentially by several epigenetic factors. The HLA gene expression levels of HLA genes can have crucial effects in the pathogenesis of diseases. Thus, detection of SNPs, insertion and deletions located outside of exons, could be critical. We now know that expression of HLA-C loci is dependent on sequences in the non-coding region of the HLA-C gene [93]. We also have learned that cancer patients' HLA genes undergo mutations, and loss of heterozygosity (LOH) facilitating the tumor to constantly evade the immune surveillance. With the implementation of NGS platform for HLA molecular typing we are poised to obtain pertinent information in terms of exact HLA matching using the 2, 3 and 4 fields with respect to sense mutations outside of the peptide binding groves and also about potential levels of expression of various HLA alleles. In the near future, we will be learning more in terms of exact matching using 3 and 4 fields. Also, we will have the ability to sequence the C4 genes of MHC-III or the entire MHC-III region for SNPs using the NGS. If phase defined complete sequencing of HLA genes, including functional and regulatory regions, is performed, novel mismatched alleles associated with GVHD in otherwise HLA matched HSCT might be identified. Furthermore, knowing the exact HLA alleles could lead to deciphering the pharmacogenomics of certain drugs that are used in conventional therapeutic regimens of GVHD. Also, clear HLA allelic resolution and the identification of constantly changing mHA profile will help us to use that information to apply to appropriate T cell based therapeutics against GVHD. To date, most of the literature on GVHD assessments are coming from programs that used non-NGS based sequencing or intermediate resolution SSP/SSOP based molecular HLA typing method for unrelated donors; technologies that are not fully capable of resolving the HLA alleles between the recipient/donor pairs especially in unrelated donor transplantation. This opens up a caveat regarding how perfect the HLA matching was between the recipient/donor pair. Molecular typing strategies other than the NGS technologies (chemistry and software) cannot handle the complexities of the HLA loci in terms of heterozygosity/homozygosity and extensive polymorphisms. Using the NGS based HLA typing, we have the ability to connect to the tight linkage disequilibrium due to multiple reads, increased read lengths, throughput, accuracy as well as development of new bioinformatics tools- now enabling us to efficiently generate complete and accurate full-length HLA haplotypes without ambiguities.

In addition, with the latest reagents and software, sequencing using NGS can provide a thorough analysis of all classical (A, B, C, DRB1, DRB3/4/5, DQA1, DQB1, DPA1, DPB1) and non-classical HLA loci (E, F, G H) and MICA /MICB. Improved chemistry and software, could help us better evaluate the DP mismatch in terms of expression and permissiveness/non-permissiveness in GVHD or HVGD direction. The new NGS technology could also help us to explore SNPs in C4/MHC III region, and identify SNPs that contribute towards mHA variations. This information may guide us towards developing T cell / B cell based therapeutics that could lead to better strategies in managing GVHD.

Furthermore, we are learning more about antibody mediated GVHD which could be due to antibodies against the mismatched HLA antigens by the donor's memory B cells, or as per the latest reports, could also be due to the antibodies against non-HLA antigens of the recipients' somatic cells. While the polymorphisms in non-HLA antigens are still under investigation, from the immunological point of view these non-HLA antigens could be immunogenic regardless of polymorphisms. As we know, conditioning regimens prior to HSCT could cause inflammatory injuries to various organs in the recipients that could have altered self or exposed cryptic antigens on the cell surfaces towards which the engrafted donor B cells and T cells could respond.

Conclusion

The incidence of GVHD remains a major undesired outcome in HSCT despite conventional HLA matching. With the establishment of NGS for human genome analysis, several new parameters are emerging in terms of genetics based compatibility of recipient/donor pairs for HSCT. Matching at C4 in the MHC Class III region can be a milestone towards the prevention of GVHD and an exciting area for further research. Additionally, epigenetic factors/mechanisms influencing expression of various immune response genes in the MHC Class III region in general, including C4 genes, particularly in connection to the SNPs observed, needs

to be understood as well. It is important to note that even with fully compatible HLA transplants by NGS, mHA related GVHD, and B cell based GVHD may occur. Utilizing new approaches, such as NGS, to better understand the 3' UTRs of HLA genes in terms of control of expression, and characterization of HLA-DP mismatches in terms of permissive and non-permissive, could lead to the development of improved algorithms in recipient/donor matching for HSCT. Newer technologies to detect non-HLA antibodies with high sensitivity that might broaden the contribution of B cell/antibody mediated GVHD have been developed. There are pointers towards targeted therapeutics to deal with these. With these recent technological advancements we are closing in towards having the tools to understand the immunopathogenesis of GVHD that could lead to newer target oriented therapeutics and potentially better GVHD outcomes.

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