GENETIC VARIANTS AS TARGETS FOR IMMUNOTHERAPY OF HEMATOLOGICAL TUMORS

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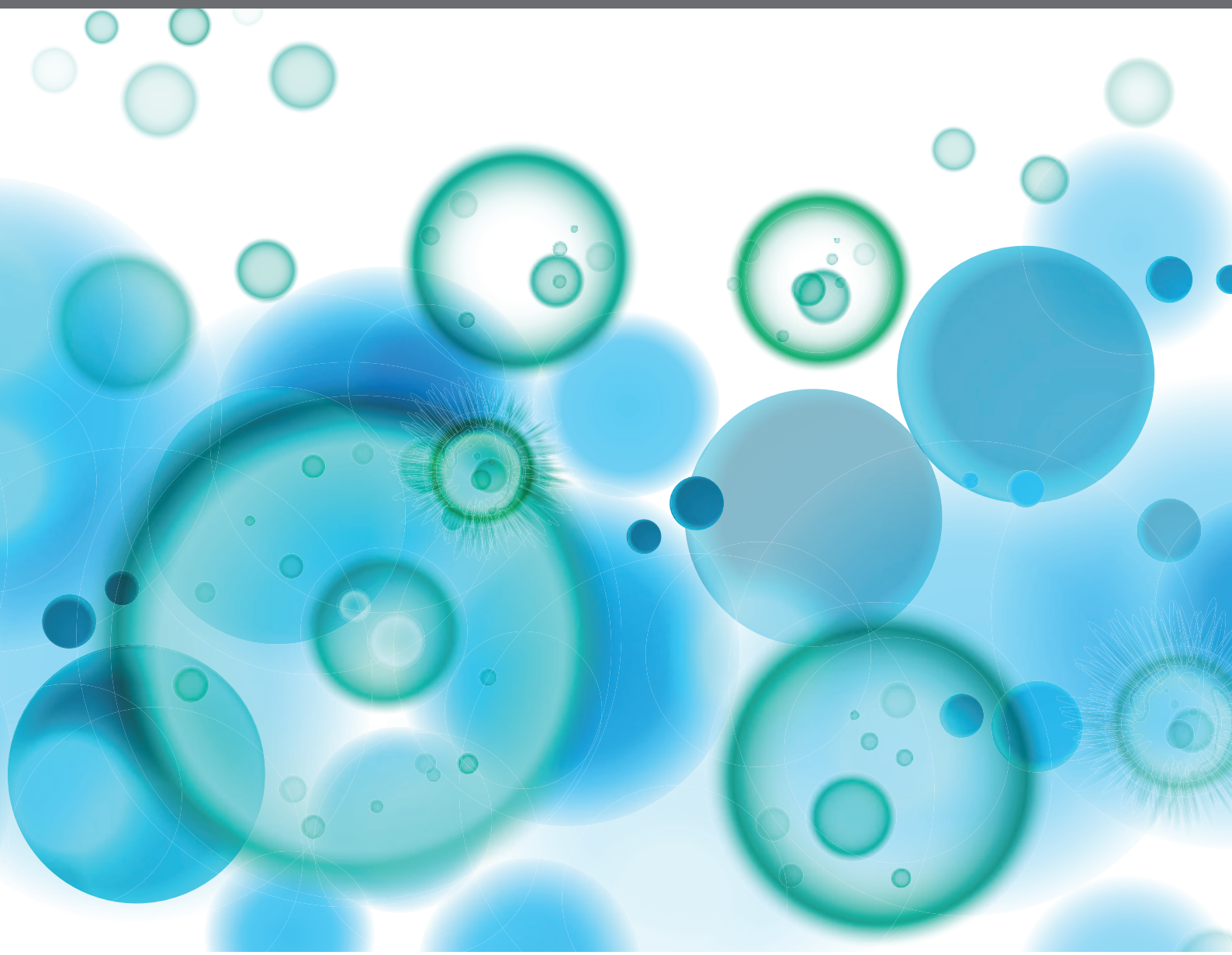

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GENETIC VARIANTS AS TARGETS FOR IMMUNOTHERAPY OF HEMATOLOGICAL TUMORS

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Neoantigens in Hematological Malignancies—Ultimate Targets for Immunotherapy?

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Neoantigens derive from non-synonymous somatic mutations in malignant cells. Recognition of neoantigens presented via human leukocyte antigen (HLA) molecules on the tumor cell surface by T cells holds promise to enable highly specific and effective anti-cancer immune responses and thus neoantigens provide an exceptionally attractive target for immunotherapy. While genome sequencing approaches already enable the reliable identification of somatic mutations in tumor samples, the identification of mutation-derived, naturally HLA-presented neoepitopes as targets for immunotherapy remains challenging, particularly in low mutational burden cancer entities, including hematological malignancies. Several approaches have been utilized to identify neoepitopes from primary tumor samples. Besides whole genome sequencing with subsequent *in silico* prediction of potential mutation-derived HLA ligands, mass spectrometry (MS) allows for the only unbiased identification of naturally presented mutation-derived HLA ligands. The feasibility of characterizing and targeting these novel antigens has recently been demonstrated in acute myeloid leukemia (AML). Several immunogenic, HLA-presented peptides derived from mutated Nucleophosmin 1 (NPM1) were identified, allowing for the generation of T-cell receptor-transduced $NPM1^{mut}$-specific T cells with anti-leukemic activity in a xenograft mouse model. Neoantigen-specific T-cell responses have also been identified for peptides derived from mutated isocitrate dehydrogenase (IDH^{mut}), and specific T-cell responses could be induced by IDH^{mut} peptide vaccination. In this review, we give a comprehensive overview on known neoantigens in hematological malignancies, present possible prediction and discovery tools and discuss their role as targets for immunotherapy approaches.

Keywords: neoantigens, hematological malignancies, mass spectrometry, immunopeptidomics, HLA antigens, NPM1 mutations

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INTRODUCTION

Recognition of tumor-associated antigens via human leukocyte antigen (HLA) molecules is pivotal for T cell-mediated tumor control and the induction of anti-tumor responses by immunotherapy (1). Neoantigens derive from non-synonymous somatic mutations and are of special interest, as they entail optimal tumor-specificity and lack central T-cell tolerance (2). These potentially highly

immunogenic antigens are therefore considered prime targets for immunotherapy, particularly since neoantigens were described as targets of immune checkpoint inhibitor-induced anti-tumor T-cell responses (3, 4, 4–6). Recent advances in mass spectrometry (MS) (7, 8) and HLA antigen prediction algorithms (9–14) as well as the broad availability of whole genome sequencing (WGS) portrayed milestones in the field of cancer immunotherapy and hold promise to enable a robust and personalized identification of neoantigens in the future. The identification of spontaneous, neoantigen-specific T-cell responses in patients with long-lasting remissions suggests that neoantigen-specific targeting of tumor cells might enable durable anti-tumor responses (15–17). Long-lasting remissions could also be observed after personalized neoantigen-based peptide vaccination therapy in melanoma patients. Keeping the small sample size in these studies in consideration, these reports further indicate toward a therapeutic potential of neoantigens (18, 19). Following the success of checkpoint inhibitor therapy and the uncovering of the specificities of respective T-cell responses, a multitude of HLA-presented neoantigens have been identified in high mutational burden diseases such as melanoma (1, 3, 20). Hematological malignancies (HM) on the other hand are characterized by a low mutational burden and the role of neoantigens for immune-mediated tumor control and immunotherapeutic approaches in these entities remains to be elucidated. The immunogenicity of acute myeloid leukemia (AML) and other HM is demonstrated by the graft-vs.-leukemia/lymphoma effect and despite the typically low mutational burden, there has been steady progress in the identification of targetable neoantigens in these diseases in recent years. In this review we give an overview on known neoantigens in HM, their means of identification as well as the current state of efforts regarding the translation of these discoveries into the clinic.

IDENTIFICATION OF PATIENT-SPECIFIC NEOANTIGENS

While genome sequencing approaches already allow for the reliable identification of patient-individual, tumor-specific mutations (1, 21), the subsequent identification of mutation-derived neoantigens remains challenging (1, 22). These novel targets can be present as mutated membrane proteins or, more frequently, as HLA-presented peptide fragments derived from intracellular proteins comprising the mutated sequence (1, 22). Frequently, identification of these HLA-presented neoepitopes is performed by *in silico* prediction of potential HLA binding motifs based on identified somatic mutations and subsequent confirmation of immunogenicity in *in vitro* T-cell assays by priming of naïve T cells or demonstration of pre-existing memory T-cell responses (1). However, as there is no direct correlation between genome, transcriptome, and immunopeptidome (23–25), this "reverse immunology approach" based on gene expression data and *in vitro* analyses can provide several "false positive" neoantigens lacking natural presentation on the tumor cell surface (18). This lack of correlation between gene expression and the immunopeptidome can be explained by the complex process of HLA ligand formation, which is furthermore frequently altered in tumor cells (26–29). Thus, only a very small fraction of predicted neoantigens is actually naturally processed and presented via HLA molecules on the tumor cell, calling for direct identification methods of HLA-presented neoepitopes to identify suitable targets for immunotherapy. This can be achieved by MS-based immunopeptidomics, which enables the only unbiased, in-depth analysis of the naturally presented HLA immunopeptidome (8, 30). Recent reports estimate, that only approximately one mutation-derived HLA-presented neoepitope arises from about 1,000 non-synonymous mutations (18, 22, 31–34). In HM, which are typically low mutational burden diseases with only a handful to a few hundred mutations (20), this implicates a low abundance or even absence of HLA-presented neoepitopes. Considering further that these can derive from passenger mutations, which are sensitive to immune escape mechanisms (1, 22) and are mainly patient-specific, the presence of broadly targetable neoantigens cannot be taken for granted in these diseases. Nevertheless, identification and successful targeting of recurrent and mainly driver mutation-derived neoantigens has recently been demonstrated in various HM (35–49) (**Table 1**), thereby expanding the prospects for immunotherapy in these entities.

NEOANTIGENS IN ACUTE MYELOID LEUKEMIA

In AML, the mutational landscape is well-characterized (64) and several novel antigens derived from recurrent genetic alterations have been identified recently. Neoantigens derived from Nucleophosmin 1 mutations ($NPM1^{mut}$), which occur in about 35% of AML patients (65), are arguably the most prominent targets in this regard. In most cases of $NPM1^{mut}$ AML, a frameshift mutation in exon 12 leads to an altered c-terminal protein sequence, which can specifically be recognized by cytotoxic $CD8^+$ T cells (66). It has been proposed, that the immunogenicity of $NPM1^{mut}$ neoepitopes might add to the favorable prognosis of AML patients with NPM1 mutations (39). Several $NPM1^{mut}$-derived HLA class I neoepitopes were identified by MS analysis in two recent studies (46, 50) and specific T-cell responses could be demonstrated in respective patients. Furthermore, isolation and transfer of a T-cell receptor (TCR) gene with an $NPM1^{mut}$ neoepitope specificity was performed in one of these studies. TCR-transduced T cells subsequently showed anti-tumor efficacy in an AML xenograft mouse model, thereby emphasizing the potential of $NPM1^{mut}$-specific T-cell-based immunotherapy approaches for the treatment of $NPM1^{mut}$ AML (46). Missense mutations of isocitrate dehydrogenase (IDH) 1 or 2 can be detected in about 20% of AML patients, resulting in an altered, leukemogenesis promoting function of the enzymes (67). A study in glioma patients, where IDH mutations occur particularly frequent, identified an $IDH1^{mut}$-derived HLA class II neoepitope and demonstrated its natural presentation and immunogenicity by detection of spontaneous $CD4^+$ T-cell responses and

TABLE 1 | Overview of neoantigens in hematological malignancies.

Hematological malignancy	Source protein of mutated neoantigen	Identification method	References
AML	NPM1	MS, spontaneous $CD8^+$ T-cell responses	(46, 50, 51)
	IDH 1	Spontaneous $CD4^+$ T-cell responses	(44)
	IDH 2	MS	(51)
	FLT3	Spontaneous $CD8^+$ T-cell responses	(38, 43)
	PML-RARα, DEK-CAN, ETV6–AML1	*In vitro* T-cell recognition	(52–54)
	Splice variants: NOTCH2, FLT3, CD44	Identification of transmembrane proteins	(35, 36)
CLL	ALMS1, C6ORF89, FNDC3B	Spontaneous $CD8^+$ T-cell responses	(17)
CML	BCR-ABL	MS, spontaneous $CD8^+$ T-cell responses	(41, 42, 55–59)
MCL	Ig heavy/light chain	MS, spontaneous $CD4^+$ T-cell responses	(60)
MPN	JAK2	*In vitro* T-cell recognition	(48)
	CALR	*In silico* prediction, spontaneous $CD4^+$ T-cell responses	(45, 47, 49, 61, 62)
	MPL	*In silico* prediction	(63)
Various	FBXW7	Spontaneous $CD8^+$ T-cell responses	(45)
	MYD88	*In silico* prediction, *in vitro* T-cell recognition, spontaneous $CD8^+$ T-cell responses	(40)

AML, acute myeloid leukemia; ALMS1, Alstrom syndrome protein 1; CALR, calreticulin; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; C6ORF89, chromosome 6 open reading frame 89; FBXW7, F-box/WD repeat-containing protein 7; FLT3, FMS like tyrosine kinase 3; FNDC3B, fibronectin type III domain containing 3B; IDH, isocitrate dehydrogenase; Ig, immunoglobulin; JAK2, janus like kinase 2; MCL, mantle cell lymphoma; MPL, myeloproliferative leukemia virus oncogene; MPN, myeloproliferative neoplasia; MS, mass spectrometry; MYD88, myeloid differentiation primary response protein 88.

mutation-specific antibodies in respective patients. $CD4^+$ T-cell responses and mutation-specific antibody formation were subsequently induced by peptide vaccination in an HLA-humanized mouse model and led to $IDH1^{mut}$-specific immune responses (44). Our own data support $NPM1^{mut}$- and IDH^{mut}-derived neoepitopes as promising targets in AML. Using our MS-based immunopeptidomics approach (24, 68–70), we were able to identify naturally presented HLA class I and II neoepitopes derived from mutated NPM1 and IDH2 in primary AML samples. Further analysis revealed multifunctional T-cell responses, and peptide-specific target cell killing was proven for one naturally presented $NPM1^{mut}$ neoepitope (51). Additionally, AML-specific neoantigens can arise from internal tandem duplications (ITD) of the FMS like tyrosine kinase 3 (FLT3) gene, which occur in up to 30% of AML patients (71). While these duplications vary in length, the same protein domain is affected in the majority of cases (72). These mutations can yield immunogenic peptides, as described for a FLT3-ITD-derived HLA-A*01:01-restricted neoepitope showing specific T-cell responses *in vitro* as well as *ex vivo* (38, 43). A further source of HLA-presented neoepitopes are fusion proteins. In AML, *in vitro* T-cell recognition of fusion protein-derived HLA-presented peptides has been demonstrated for PML-RARα (52), DEK-CAN (53), and ETV6–AML1 (54). While these reports arouse interest in these potential targets, the clinical significance of these *in vitro* analyses remains to be elucidated as natural presentation and spontaneous immune responses against respective HLA-presented neoepitopes have not been demonstrated.

NEOANTIGENS IN CHRONIC MYELOID LEUKEMIA AND MYELOPROLIFERATIVE DISORDERS

In chronic myeloid leukemia (CML), peptides encompassing the BCR-ABL fusion site in theory represent optimal targets for immunotherapy, as this fusion protein is essential for the malignant transformation, is present in virtually all CML cells and patients, and potentially provides HLA binding motifs. One major throwback however is the occurrence of several different fusion sites resulting in diverse mutation-derived peptides in distinct patients. The *t*(9;22) translocation mainly leads to the formation of an exon junction between exon 2 or 3 of *BCR* and exon 2 of *ABL* (*b2a2* and *b3a2,* respectively) (73). Several groups have described specific T-cell responses against HLA-presented peptides derived from b3a2 (41, 42, 55, 56). Furthermore, it has been demonstrated that BCR-ABL-specific T-cell responses can be induced with peptide vaccination in CML patients (57). One study reported the direct identification of a HLA-presented b3a2-derived neoepitope by MS on primary CML cells (58). However, in our recently performed extensive MS-based analysis of the primary CML immunopeptidome, we could not identify any naturally HLA-presented peptides encompassing BCR-ABL- or ABL-BCR-derived neoepitopes (68), keeping in mind that especially for MS-based immunopeptidomics, absence of evidence does not equal evidence of absence. Notably, neoantigens can also arise under therapy with tyrosine kinase inhibitors. In patients with imatinib-resistant CML, drug

resistance-mediating mutations outside the BCR-ABL fusion site have been identified (59) and specific T-cell responses against neoepitopes derived from these mutations have been demonstrated and were linked with clinical response (59). Myeloproliferative disorders (MPN) are characterized by a homogenous mutational landscape with recurrent driver mutations, which in theory represent shared and therefore broadly applicable targets for immunotherapy. A single nucleotide mutation of the janus kinase 2 gene (JAK2 V416F) is the most frequent among MPN driver mutations, occurring in more than 90% of patients with Polycythemia vera (PV) and about 50% of patients with Essential thrombocytosis (ET) and Primary myelofibrosis (PMF), respectively (74). While experimental recognition of $JAK2^{mut}$-derived neoepitopes has been demonstrated after *in vitro* priming of healthy donor T cells, thereby indicating the immunogenic potential, no spontaneous T-cell responses have been identified in $JAK2^{mut}$ MPN patients (48). Direct identification of naturally presented $JAK2^{mut}$ neoepitopes has not been reported so far. In MPN with Calreticulin mutations ($CALR^{mut}$)—the most common driver mutation in JAK2 wildtype ($JAK2^{wt}$) MPN, occurring in about 25% of ET and PMF patients (74)—a frameshift mutation leads to an altered c-terminus of the protein. Recent reports evaluated $CALR^{mut}$-derived HLA-presented neoepitopes as targets for immunotherapy (45, 47, 49, 61, 62) as these peptides were predicted to bind HLA-A*03:01 and HLA-B*07:02. While natural presentation of these HLA class I neoepitopes could not be demonstrated upon MS analysis (45), spontaneous, primarily $CD4^+$ T-cell responses against several $CALR^{mut}$-derived neoepitopes could be identified in $CALR^{mut}$ MPN patients (49). Further, $CALR^{mut}$-dependent killing of autologous $CALR^{mut}$ cells was demonstrated in an HLA-DR-restricted manner (47). Of note, while the observed $CALR^{mut}$-specific T-cell responses in $CALR^{mut}$ MPN patients were often weak (45, 49, 61), these could be restored by immune checkpoint blockade both *in vitro* and *in vivo* (61). These findings indicate that $CALR^{mut}$ is naturally presented but respective T-cell responses are suppressed by immune checkpoint receptor signaling (61), illustrating the potential of combining distinct immunotherapeutic approaches for the treatment of HM. Taken together, the results revealed $CALR^{mut}$-derived neoepitopes as shared MPN-specific neoantigens, prompting their further evaluation for therapeutic targeting (47, 49, 61, 62). Interestingly, $CALR^{mut}$-specific memory T-cell responses were frequently detectable in healthy individuals in a subsequent study, suggesting a previous clearance of $CALR^{mut}$ cells by immunosurveillant T cells and thereby further highlighting the immunogenic potential of $CALR^{mut}$ neoantigens (62). Mutations in the myeloproliferative leukemia virus oncogene (MPL) are further recurrent driver mutations in $JAK2^{wt}$ MPN (75). A recent study performing an *in silico* analysis based on whole transcriptome sequencing of MPN patients predicted several MPL^{mut} HLA class I binding neoepitopes (63). However, demonstration of natural presentation of MPL^{mut} neoepitopes by MS has not been performed thus far.

NEOANTIGENS IN OTHER HEMATOLOGICAL MALIGNANCIES

FBXW7 is a tumor suppressor gene with mutations occurring in various HM, most frequently in T-ALL (76). Specific $CD8^+$ T-cell responses against a recurrent $FBXW7^{mut}$-derived neoepitope have been demonstrated, suggesting that this recurrent mutation might represent another neoantigen applicable for immunotherapy in HM (45). Mutation-derived neoantigens have also been identified in chronic lymphocytic leukemia (CLL). In a study, implementing a reverse immunology approach, immune responses were detected against HLA antigens derived from somatic mutations in ALMS1, C6ORF89, and FNDC3B (17). However, it should be kept in mind that these mutations are not considered driver mutations, thus theoretically making them suboptimal targets for immunotherapy. The identification of T-cell responses against these mutated neoantigens nevertheless demonstrated the applicability of neoantigen-specific targeting of CLL. Evaluation of neoantigens in mantle cell lymphoma patients applying a combined approach of whole exome sequencing and direct HLA ligand identification by MS revealed the presence of naturally presented HLA class II neoepitopes derived from the lymphoma immunoglobulin heavy- or light-chain variable regions (60). Spontaneous $CD4^+$ T-cell responses could be identified against these neoepitopes and mediated tumor-specific killing of autologous lymphoma cells (60). MYD88 (L265P) is a recurrent driver mutation in Waldenstom's macroglobulinemia, CLL and other Non-Hodgkin lymphomas (77). We previously evaluated HLA class I neoepitopes derived from $MYD88^{mut}$ as targets for immunotherapy in lymphoma patients (40). Based on *in silico* HLA motif prediction, further immunogenicity evaluation of possible $MYD88^{mut}$-derived HLA class I neoepitopes was performed. *In vitro* priming of naïve T cells from $MYD88^{mut}$ CLL patients and healthy donors was successful for several HLA-B:07- and HLA-B:15-restricted neoepitopes. While further analysis revealed that spontaneous $MYD88^{mut}$-specific T-cell responses are infrequent in lymphoma patients, these *de novo* induced $MYD88^{mut}$-specific T cells were multifunctional and elicited mutation-restricted cytotoxicity (40), highlighting the potential of $MYD88^{mut}$ neoepitopes as targets for immunotherapy.

NON-CANONICAL NEOEPITOPES AS ADDITIONAL TUMOR-SPECIFIC TARGETS

While the term *neoantigen* is mostly used referring to mutation-derived HLA-presented neoepitopes, the following section will discuss further sources of neoantigens that might also represent promising targets for immunotherapy. In distinction to mutations of the target's primary genome sequence, non-canonical neoantigens or cryptic peptides arise among others from tumor-specific alterations of the HLA antigen presentation machinery, DNA methylation, RNA editing or protein biosynthesis, proteasomal splicing, or non-canonical

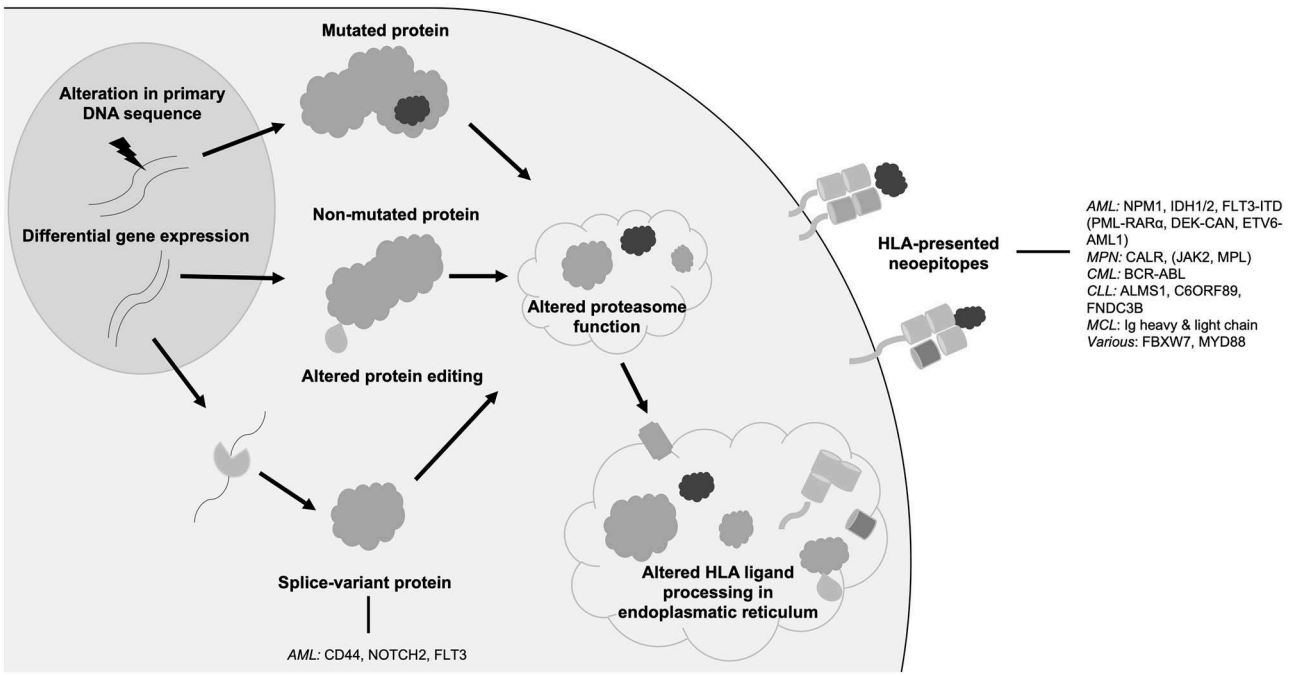

FIGURE 1 | Origins of neoantigens. Schematic overview showing different origins of neoantigens with prominent examples in hematological malignancies. AML, acute myeloid leukemia; FLT3, FMS like tyrosine kinase 3; HLA, human leukocyte antigen; NPM1, nucleophosmin 1; IDH, isocitrate dehydrogenase; MPN, myeloproliferative neoplasia; CALR, calreticulin; JAK2 janus like kinase 2; CML, chronic myeloid leukemia; CLL, chronic lymphocytic leukemia; MCL, mantle cell lymphoma; Ig, immunoglobulin.

translation products (**Figure 1**) (29, 78–83). Further, tumor-specific splice variant proteins can result from splice-site creating mutations or mutations of spliceosome proteins and can lead to the formation of tumor-specific HLA-presented neoepitopes (84). Mutations directly affecting the spliceosome, such as SF3B1 or SRSF2 mutations, have been shown to occur in up to 20% of *de novo* AML (64) and 15% of PMF (63). In AML, frequently occurring splice variants have been identified for NOTCH2 (35), FLT3 (35), and CD44 (36), each leading to the occurrence of an altered cell surface protein. Specific targeting of a CD44 splice variant has been demonstrated by a CD44v6 CAR T cell in a mouse model (36), thereby highlighting the potential of targeting this class of neoantigens with immunotherapeutic approaches. Post-translational protein modifications, which are preserved in HLA-presented peptides, can portray another source of neoantigens as these tumor-specific alterations can lead to the formation of novel epitopes (29). In this regard, neoantigens resulting from tumor-specific phosphorylation as well as glycosylation have been identified in AML and immunogenicity of this class of neoantigens has been demonstrated (28, 37, 85, 86).

DISCUSSION

Neoantigen targeting holds promise to enable highly specific and durable anti-tumor immune responses (1, 22). Although HM are typically low mutational burden diseases (20), there has recently been remarkable progress in the uncovering of neoantigens in these entities. These discoveries were fueled by an immense progress in the field of WGS, steadily improved HLA motif prediction algorithms as well as technical advances in MS in recent years (1, 21, 31). While these advances already facilitate neoantigen identification from primary tumor samples, we are likely only seeing the beginning of personalized target evaluation. In this progress, a standard approach for target identification has yet to be defined (1, 22). As optimal target selection is a prerequisite for effective immunotherapy, we consider the direct identification of HLA-presented neoantigens by MS as the optimal approach. The direct identification of potential targets with MS harbors essential advantages when compared to the reverse immunology approach, which relies on neoantigen prediction, experimental HLA-binding and further immunogenicity confirmation in T-cell assays (1, 22). Here, "false targets" might be identified if HLA binding is demonstrated for predicted neoantigens *in vitro*, but these antigens are not naturally presented via HLA antigens *in vivo*. Furthermore, while the *ex vivo* identification of spontaneous T-cell responses against neoantigens can be regarded as evidence for natural HLA-presentation, potential targets without pre-existing responses might be missed. This is concerning as antigen-specific immunotherapy, including antibody strategies and peptide vaccines in particular aim to induce *de novo* anti-tumor responses. HM have been the first entities where immunotherapy—in form of allogenic stem cell transplantation (87)—has been performed and the immunogenicity of HM is long known (88, 89). While targeting of mutated membrane

proteins by antibodies or CAR T cells has already been established for the treatment of HM, but is restricted to very few suitable surface antigens (90–94), HLA-presented neoantigens derived from intracellular proteins are of particular interest for immunotherapy. Hence, recent highly noted reports on the identification of neoantigens in HM have raised hopes that these novel targets might bring along new therapeutic options. In AML, $NPM1^{mut}$- and IDH^{mut}-derived neoantigens thus far represent the most promising targets, as these mutations occur frequently and successful as well as specific targeting has already been demonstrated in preclinical studies (44, 46, 51). Neoepitopes derived from fusion proteins are equally interesting targets, but natural presentation via HLA molecules has not been demonstrated so far (41, 42, 52–54, 56, 57, 95). While adoptive T-cell transfer and peptide vaccination approaches using non-mutated antigens are already under clinical evaluation in AML and other HM (96–98), the identification of these neoantigens will allow for an even more targeted approach in the future. However, it should be kept in mind that a personalized target selection remains challenging, as MS analysis is elaborate and not universally available. Furthermore, completely personalized immunotherapy approaches at the same time bring the difficulty of manufacturing an individualized product, e.g., a peptide vaccine, for each patient. A "warehouse" model, where a patient-specific selection of therapeutics targeting frequently occurring neoantigens can be made, might represent an elegant solution to this problem. This is particular true for malignancies with a well-characterized mutational landscape and a narrow spectrum of recurrent mutations, such as AML and MPN (20). At the same time and despite the recent progress in neoantigen identification in HM, the infrequency of individual mutations and HLA allotype restrictions still limit specific neoantigen targeting to a subset of patients. To overcome this issue, combined targeting of both mutated and non-mutated, tumor-exclusive antigens might be a suitable approach. Tumor-exclusive non-mutated neoepitopes can arise as a consequence of differential gene expression or tumor-specific alterations of RNA- and protein-processing, as described for splice variant proteins in AML (35, 36, 64) and have been shown to possess equally immunogenic properties as mutated neoepitopes (99). With mutations of spliceosome proteins, transcription factors and DNA methylation related proteins occurring frequently in AML and other HM (20, 64), additional non-canonical neoantigens are likely awaiting uncovering (35, 36, 84). Recent neoantigen discoveries have created novel and promising prospects for immunotherapy in HM. With currently ongoing endeavors, additional neoantigens might be uncovered and personalized target evaluation will be taken another step further. Considering the paucity of targetable mutated neoantigens in the individual patient due to HLA allotype restrictions and patient-individual mutations, combined approaches targeting both mutated and non-mutated tumor-exclusive antigens are likely warranted in patients with HM. Harnessing "the best of both worlds" might then enable immunotherapy to unfold its full potential in hematological malignancies.

AUTHOR CONTRIBUTIONS

MR wrote the first draft of the manuscript. AN and JW wrote further sections of the manuscript. All authors contributed to manuscript revision, read, and approved the final version.

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The Genomic Landscape of Antigenic Targets for T Cell-Based Leukemia Immunotherapy

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Intensive fundamental and clinical research in cancer immunotherapy has led to the emergence and evolution of two parallel universes with surprisingly little interactions: the realm of hematologic malignancies and that of solid tumors. Treatment of hematologic cancers using allogeneic hematopoietic cell transplantation (AHCT) serendipitously led to the discovery that T cells specific for minor histocompatibility antigens (MiHAs) could cure hematopoietic cancers. Besides, studies based on treatment of solid tumor with *ex vivo*-expanded tumor infiltrating lymphocytes or immune checkpoint therapy demonstrated that anti-tumor responses could be achieved by targeting tumor-specific antigens (TSAs). It is our contention that much insight can be gained by sharing the tremendous amount of data generated in the two-abovementioned universes. Our perspective article has two specific goals. First, to discuss the value of methods currently used for MiHA and TSA discovery and to explain the key role of mass spectrometry analyses in this process. Second, to demonstrate the importance of broadening the scope of TSA discovery efforts beyond classic annotated protein-coding genomic sequences.

Keywords: genomics, major histocompatibility complex, mass spectrometry, minor histocompatibility antigen, peptide, proteogenomics, RNA sequencing, tumor-specific antigen

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INTRODUCTION—CLASSIFICATION OF ANTIGENIC TARGETS

MHC-associated peptides (MAPs) are by-products of protein degradation by proteasomes and other proteases (1). However, while all proteins ultimately undergo proteolytic degradation, only some of them generate MAPs (2, 3). Indeed, the biogenesis of MAPs is regulated by several mechanisms operating at the transcriptional, translational, and post-translational levels (4, 5). Notably, MAPs preferentially derive from proteins degraded during or in the minutes following translation, perhaps by specialized "immunoribosomes" (6).

Four groups of MAPs can be targeted for T-cell based immunotherapy of hematologic cancers: MiHAs, tumor-associated antigens (TAAs), mutated TSAs (mTSAs), and aberrantly expressed TSAs (aeTSAs). MiHAs are encoded by genomic regions with two cardinal features: they contain germline polymorphisms, and they are expressed in both normal and neoplastic cells (7, 8). TAAs derive from unmutated genes that are expressed in normal cells but are overexpressed in cancer cells. In several studies, TAAs have been defined according to the overexpression of the corresponding RNA or source protein. This criterion is not entirely satisfactory considering that (i) T cells see MAPs, not RNA or proteins, and (ii) there is no linear correlation between the abundance of MAPs and the abundance of their source RNA or protein (9–11). Ideally, TAAs should therefore be defined according to MAP abundance on normal vs. neoplastic cells. TSAs are MAPs present only on cancer cells. Identification of mTSAs is relatively straightforward: these MAPs are coded

by transcripts bearing somatic mutations such as single nucleotide variants, fusion transcripts, etc. (12, 13). Identification of aeTSAs is more challenging since they are unmutated MAPs that can arise from any genomic region *via* cancer-specific aberrations in gene expression (e.g., alterations in histone or DNA methylation) or splicing (14–17).

Identification of aeTSAs rests on the demonstration that these unmutated MAPs are present only on cancer cells. Two strategies have been used to achieve this goal. The first one hinges on comparison of the immunopeptidome (MAP repertoire) of cancer cells *vs*. that of normal cells (18–20). MAPs found only on cancer cells following mass spectrometry (MS) analyses are labeled as cancer-specific. The limitation of this approach is that some putative aeTSAs may not be entirely cancer-specific because it is currently impossible to obtain the entire MAP repertoire of all types of normal cells. This is particularly true for medullary thymic epithelial cells (mTECs) which have a unique ability to promiscuously express more genes than other types of somatic cells (21). For example, mTECs express several TAAs, that would otherwise qualify as aeTSAs, such as MAGE-A1, MAGE-A3, MAGE-A4, NY-ESO, and CEA (22). Since mTECs induce central immune tolerance, MAPs expressed in mTECs are expected to be poorly immunogenic. It has heretofore been impossible to analyze the immunopeptidome of mTECs because the number of mTECs that can be obtained from a human subject [$\approx 10^6$ cells (23)] is inferior to the number required for comprehensive MS analyses ($\approx 10^8$ cells) and mTECs cannot be expanded *ex vivo*. The second strategy is based on the simple principle that a MAP cannot be present if its source RNA is not expressed. Accordingly, MAPs identified in cancer cells by MS analyses are labeled as aeTSAs only when their source RNA is not expressed in any tissue or organ, including mTECs (14, 16). A caveat of this approach is that presence of a MAP-coding RNA is necessary but not sufficient for expression of this MAP at the peptide level. Hence, this strategy may be too stringent and discard some *bona fide* aeTSAs that would be cancer-specific at the peptide but not the RNA level.

IDENTIFICATION OF TUMOR-SPECIFIC ANTIGENS

Since the focus of this series is on genetic variants, we will concentrate on TSAs and MiHAs for the rest of this article. This does not mean that TAAs are not interesting targets. The main caveat of TAAs is that they are expected to be poorly immunogenic because they are seen as self-MAPs by T cells. However, transfection of CD8 T cells with a high-affinity WT1-specific TCR yielded promising results in a seminal trial on prevention of AML relapse after allogeneic hematopoietic cell transplantation (24). Notably, no off-target toxicity was observed despite the fact that WT1 is expressed by hematopoietic stem cells, urogenital epithelia, and by mesothelial and fibroblastic cells of the peritoneum, the pleural cavity, and the pericardial cavity (24, 25). Moreover, a vaccine targeting the PR1 TAA also induced PR1-specific immune response in patients with myeloid malignancies (26). Nonetheless, the majority of clinical trials involving TAAs have shown a limited therapeutic potential (27, 28). In contrast to TAAs, TSAs, and MiHAs represent non-self MAPs for autologous and allogeneic T cells, respectively (16, 29, 30). We will limit our review to TSAs and MiHAs presented by MHC class I molecules because the number of studies on MHC II MAPs is relatively limited.

Many studies have been performed in search of TSAs in various tumor types. In most cases, putative TSAs (aka neoantigens) have been identified based on exome sequencing and algorithms that predict MHC binding, without MS validation. This approach is fraught with two major caveats: limited scope and low accuracy.

Limited Scope

Exons represent only 2% of the genome, whereas 75% of the genome can be transcribed and potentially translated (31). Indeed, MS analyses identified MAPs derived from all sorts of allegedly non-protein-coding regions: introns, 5′UTRs, 3′UTRs, long non-coding RNAs, and intergenic regions (14). Accordingly, many allegedly non-coding regions are in fact protein coding, and translation of "non-coding regions" has been shown to generate numerous MAPs (32–34) some of which were retrospectively identified as targets of TILs and autoreactive T cells (35, 36). In addition, the vast majority of TSAs, and of aeTSAs in particular, derive from allegedly non-coding regions (14). We estimate that mTSAs encoded by canonical exonic open reading frames represent <10% of human TSAs (14). Furthermore, the number of exonic mTSAs should be exceedingly low in leukemias because their mutational load is orders of magnitude lower than that of solid tumors such as melanoma. In fact, to the best of our knowledge, only one mTSA has been unambiguously validated by MS in acute leukemias: this HLA-A*02:01–binding peptide results from mutations in the *NPM1* gene that cause the translation of a C-terminal alternative reading frame (15). Another mTSA derived from a BCR-ABL fusion protein was identified *via* MS analyses in 2001 (37), but was not found in a larger cohort of subjects in 2019 (38), and its immunogenicity was called into question (39). The status of this putative TSA therefore remains unclear.

Low Accuracy

The story of the TEL-AML1 fusion peptide provided one of the first hints that, in the absence of MS validation, predictions based on reverse immunology could be misleading. The TEL-AML1 fusion protein results from a 12; 21 chromosomal translocation and is an important transforming factor in B-cell precursor acute lymphoblastic leukemia. Based on MHC-binding predictions, a TEL-AML1 fusion peptide that could bind to HLA-A*02:01 was identified (40). Priming of T cells against this peptide generated cytotoxic T cells that recognized autologous leukemic cells (40). However, when tested experimentally, binding of this peptide to HLA-A*02:01 was very weak and its immunogenicity very low. Furthermore, the peptide was not endogenously processed by cells because it was cleaved by proteasomes (41). Hence, the TEL-AML1 fusion peptide was a false discovery, and killing of leukemic cells by T cells primed against the TEL-AML1 fusion peptide (40) was most likely due to the

inherent cross-reactivity of T cells which is further amplified in T-cell lines (42). Indeed, positive selection in the thymus preferentially rescues cross-reactive T cells (43) and a single T-cell receptor may recognize more than a million different MAPs (44). Recently, a particularly eloquent demonstration of the low accuracy of mTSA predictions was provided by Löffler at al. who performed comprehensive multi-omic analyses of 16 primary human hepatocellular carcinomas (20). Based on exome and transcriptome sequencing data, MHC-binding algorithms predicted that individual tumors would present an average of 118 exonic mTSAs. Remarkably, none of the 1,888 predicted exonic mTSAs were detected by MS analyses (20). In view of this, the exciting claim that exonic mTSAs can be found in myeloproliferative neoplasms and childhood acute lymphoblastic leukemia must be met with enthusiasm and skepticism since no MS validation was performed on the predicted TSAs (45, 46).

How should we design TSA discovery projects in hematopoietic cancers? We propose that two elements should be taken into consideration. First, we believe that searches limited to exonic TSAs considerably underestimate the diversity of the TSA repertoire (47). According to initial analyses of primary acute lymphoblastic leukemia samples, the vast majority of TSAs are aeTSAs derived from unmutated allegedly non-coding sequences. This analysis led to the discovery that endogenous retroelements (EREs), which are part of our non-coding genome, are a rich source of TSAs. EREs can be defined as remnants of the ancient exogenous retroviruses that infected germ line cells and represent around 43% of the human genome (48). Under physiological conditions, most ERE sequences are silenced, but can be re-expressed in cancer through epigenetic dysregulation of the cancer genome (49). The expression of such sequences can lead to MHC-I presentation of "viral-like" peptides and activate T cells (50). Accordingly, our team identified three ERE-derived TSAs in human ALL samples (14). Moreover, it was shown that the env gene of HERV-K was highly upregulated in AML (51), suggesting that this gene could contribute to AML TSA landscape. Notably, since they are unmutated, aeTSAs can be shared by many patients (52, 53). Second, we strongly suggest that MS analyses should be performed either at the discovery or at the validation stage for all TSAs that might be used as therapeutic targets. Indeed, most bioinformatically "predicted TSAs" not validated by MS analyses probably represent false discoveries. This being said, MS has its own limitations (54). Actually, in the discovery mode, "shotgun MS" is biased toward the most abundant peptides and misses low abundance MAPs (55). Alternatively, targeted MS analyses decreases the detection threshold by about 10-fold, but can be performed only on a limited number of peptides of known amino acid sequence (56). Given the rapid pace of improvements in MS technology it may soon be possible to combine the breadth of shotgun MS with the sensitivity of targeted MS (11, 54).

Once TSAs are discovered, the major remaining challenge is to evaluate their immunogenicity. A recent report suggests that about 80% of virus-derived MAPs validated by MS are immunogenic in mice (57). However, we have no evidence that the rules governing immunogenicity of viral MAPs in mice will apply to TSAs in humans. We reported that the strength of anti-TSA immune response in mice was regulated by two parameters: TSA expression level and the frequency of TSA-responsive T cells in the preimmune (naïve) repertoire (14). However, since only five TSAs were studied, these data should be considered preliminary. For the time being, TSA immunogenicity cannot be predicted, and has to be tested experimentally.

IDENTIFICATION OF ACTIONABLE MINOR HISTOCOMPATIBILITY ANTIGENS

MiHAs are MAPs derived from polymorphic genomic regions. Since over 660 million single nucleotide variants (SNV) and indels have been identified in human populations (58), the potential human MiHA landscape is very broad. Even though MiHA can originate from non-synonymous SNVs in exons or in non-coding regions (32, 59, 60), we will focus herein on exonic MiHAs because they are easier to identify than those generated from atypical transcripts, and probably sufficient to enable immunotherapy of hematologic cancers. Discovery of the first MiHAs in mice (61–64) and humans (65–67) has been a major endeavor, if not a technical tour de force. However, the pace of MiHA discovery increased rapidly with progress in next generation sequencing and MS. For instance, proteogenomic studies led to the identification of over 6,000 MiHAs presented by the most common HLA haplotype in European Americans: HLA-A*02:01;B*44:03 (60). As for TSAs, MS analyses are instrumental in MiHA discovery/validation because only a small proportion of SNV generate MiHAs (59). Over 90% of MiHA loci are bi-allelic with a dominant allele (that generate MAPs) and a recessive allele (that generates no MAPs) (59, 60, 67). In a few cases, both MiHA alleles are co-dominant. Thus, if we consider MiHAs coded by dominant alleles as winners, it follows that in most cases a single SNV is sufficient to transform winners into losers (the recessive alleles). This is an eloquent reminder that we cannot predict the molecular composition of the immunopeptidome based on our limited understanding of the complexity of the MAP processing pathway (2, 59). More importantly, out of the thousands of MiHAs that we identified, only a minority represent attractive targets for immunotherapy of hematologic tumors with allogeneic T cells (60). Indeed, most MiHAs as non-actionable targets because of their low population frequency and/or their expression in normal epithelial cells.

Allelic Frequency

As long as it is expressed in tumor cells, a TSA may be considered a potential target. For MiHAs, things are more complicated: in order to be actionable, an MiHA must be present in the recipient and absent in the donor. We refer to this situation as a therapeutic mismatch. The probability to have a therapeutic mismatch is maximal when the allelic frequency of the target MiHA is 0.5 and decreases as the allele frequency approaches the two extremes of 0 and 1 (68). However, because of human population history, most bi-allelic loci have a very common and a very rare allele, with population frequencies of >0.99 and <0.01, respectively (58). MiHAs having an allele frequency of 0.01 or 0.99 would yield a

low frequency of therapeutic mismatch: in the first case, MiHA-positive recipients would be rare, whereas in the second case, MiHA-negative donors would be difficult to find. If we consider that actionable MiHA loci must have a minor allele frequency of ≥0.05, then about 92.6% of MiHAs have to be discarded (60).

Tissue Expression Profile

CD8 T cells targeted to a single MiHA can eradicate tumor cells without causing GVHD, even if expression of the target MiHA is not restricted to hematopoietic cells (69–71). Two elements provide a plausible explanation for the fact that hematopoietic cells are inherently more sensitive than epithelial cells to anti-MiHA T cells: (i) MHC molecules (and therefore MiHAs) are more abundant on hematopoietic cells than epithelial cells and (ii) in one experimental model, MiHA-specific T cells preferentially infiltrated tissues containing VCAM-1^+ microvessels, that is, the bone marrow and tumor sites (30, 70). Notably, eradication of leukemia cells cannot be achieved by targeting any MiHA. Only MiHAs recognized by CD8 T cells with high functional avidity are effective in mouse models (30, 71–74). As a corollary, we speculate that in clinical trials it may be preferable to target multiple MiHAs simultaneously. Since increasing the number of targeted MiHAs enhances the risk of GVHD (75), it would appear justified to target mainly hematopoietic MiHAs. One additional advantage of targeting non-ubiquitous MiHAs is that "antigen excess" (ubiquitous MiHAs) favor exhaustion of anti-MiHA T cells (76). As for TSAs, the question of MiHA expression by normal cells is not a trivial issue. In practice, we assessed the expression profile of MiHA-coding RNAs in normal tissues, then discarded MiHAs coded by ubiquitously expressed transcripts, and kept only MiHAs preferentially expressed in hematopoietic cells relative to epithelial cells (60). This led to the elimination of two-thirds of MiHAs. In fine, out of the 6,773 MiHAs presented by HLA-A*02:01 and HLA-B*44:03, only 39 had a minor allele frequency of ≥0.05 and an adequate tissue expression profile (60). This number was sufficient to yield at least one therapeutic mismatch in 90% of related and 98% of unrelated HLA*02:01/HLA-B*44:03-positive donor-recipient pairs (60). We conclude that the landscape of human exonic polymorphisms is vast enough for MiHA-targeted immunotherapy of practically all subjects suffering from hematologic cancers. In practice, this would require systems-level analyses of the MiHA repertoire presented by other common HLA allotypes.

TUMOR-SPECIFIC ANTIGENS AND MINOR HISTOCOMPATIBILITY ANTIGENS—TRANSLATIONAL CHALLENGES

In addition to antigen discovery *per se*, scientists involved in the development of TSA- and MiHA-targeted immunotherapies have to address two main challenges: the complexity of precision medicine and the engineering of cost-effective delivery technologies. In the case of TSAs, vaccines appear to be a reasonable delivery strategy to begin with, but the level of precision needed is not inherently obvious. On one side, advocates of individualized vaccines who focus mainly on exonic mTSAs do believe that *de novo* TSA discovery should be performed for individual patients (77, 78). Others, prefer to target shared TSAs (mainly aeTSAs) and rather foresee the development of pre-assembled multi-epitope vaccines containing a series of TSAs presented by specific HLA allotypes (16, 79). In all cases, it is imperative to improve the immunogenicity of TSA vaccines. Accordingly, several different platforms using enhanced vaccine technologies and improved co-stimulatory agents (adjuvants, superantigens, mature dendritic cells) are currently being tested for multiple tumor types including leukemia and lymphoma (28, 77, 80, 81). In the case of MiHAs, whose complexity is more limited than that of TSAs, delivery is probably the major barrier. Almost all pre-clinical research on MiHA-targeted immunotherapy has involved adoptive transfer of allogeneic T cells. Translating this into clinical practice will only be possible when we can count on reliable methods for *ex vivo* generation of sufficient numbers of fit (not exhausted) MiHA-responsive T cells (82–84). Finally, for both TSAs and MiHAs, the strength of anti-leukemic immunotherapy could be further increased with more sophisticated TCR-based therapy using transfected TCRs or bispecific biologics (24, 39, 85).

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. These data can be found here: MiHA sequences were deposited in the Immune Epitope Database (http://www.iedb.org/) under submission code 1000670. RNA-Seq and exome data were deposited in the NCBI Bioproject database (http://www.ncbi.nlm.nih.gov/bioproject/) under accession code PRJNA286122.

AUTHOR CONTRIBUTIONS

M-PH and KV: analysis and interpretation of data, final revisions of the manuscript. CP: financial support and manuscript writing.

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Conflict of Interest: Université de Montréal holds patents and has filed patent applications on minor histocompatibility antigens and tumor-specific antigens.

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Apparent Lack of *BRAF*^{V600E} Derived HLA Class I Presented Neoantigens Hampers Neoplastic Cell Targeting by CD8^{+} T Cells in Langerhans Cell Histiocytosis

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Langerhans Cell Histiocytosis (LCH) is a neoplastic disorder of hematopoietic origin characterized by inflammatory lesions containing clonal histiocytes (LCH-cells) intermixed with various immune cells, including T cells. In 50–60% of LCH-patients, the somatic *BRAF*^{V600E} driver mutation, which is common in many cancers, is detected in these LCH-cells in an otherwise quiet genomic landscape. Non-synonymous mutations like *BRAF*^{V600E} can be a source of neoantigens capable of eliciting effective antitumor CD8^{+} T cell responses. This requires neopeptides to be stably presented by Human Leukocyte Antigen (HLA) class I molecules and sufficient numbers of CD8^{+} T cells at tumor sites. Here, we demonstrate substantial heterogeneity in CD8^{+} T cell density in $n = 101$ LCH-lesions, with *BRAF*^{V600E} mutated lesions displaying significantly lower CD8^{+} T cell:CD1a^{+} LCH-cell ratios ($p = 0.01$) than *BRAF* wildtype lesions. Because LCH-lesional CD8^{+} T cell density had no significant impact on event-free survival, we investigated whether the intracellularly expressed *BRAF*^{V600E} protein is degraded into neopeptides that are naturally processed and presented by cell surface HLA class I molecules. Epitope prediction tools revealed a single HLA class I binding *BRAF*^{V600E} derived neopeptide (KIGDFGLAT**E**K), which indeed displayed strong to intermediate binding capacity to HLA-A*03:01 and HLA-A*11:01 in an *in vitro* peptide-HLA binding

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assay. Mass spectrometry-based targeted peptidomics was used to investigate the presence of this neopeptide in HLA class I presented peptides isolated from several $BRAF^{V600E}$ expressing cell lines with various HLA genotypes. While the HLA-A*02:01 binding *BRAF* wildtype peptide KIGDFGLATV was traced in peptides isolated from all five cell lines expressing this HLA subtype, KIGDFGLAT**E**K was not detected in the HLA class I peptidomes of two distinct $BRAF^{V600E}$ transduced cell lines with confirmed expression of HLA-A*03:01 or HLA-A*11:01. These data indicate that the *in silico* predicted HLA class I binding and proteasome-generated neopeptides derived from the $BRAF^{V600E}$ protein are not presented by HLA class I molecules. Given that the $BRAF^{V600E}$ mutation is highly prevalent in chemotherapy refractory LCH-patients who may qualify for immunotherapy, this study therefore questions the efficacy of immune checkpoint inhibitor therapy in LCH.

Keywords: Langerhans Cell Histiocytosis, BRAF, neoantigen, neopeptide, Human Leukocyte Antigen, T cell

INTRODUCTION

Langerhans Cell Histiocytosis (LCH) is a rare neoplastic disorder of hematopoietic origin that primarily affects children, but also involves adults (1). Its clinical manifestation varies from a single bone lesion or benign skin rash to a widely disseminated and life-threatening condition, similar to acute myeloid leukemia (2). The histopathological hallmark of LCH are phenotypically aberrant $CD1a^+$ $CD207^+$ histiocytes (LCH-cells), although not all pathological $CD1a^+$ histiocytes co-express CD207 (3). Typically, these LCH-cells are accompanied by a diverse inflammatory infiltrate, often including T cells (2). These T cells have been shown to frequently make intimate contact with LCH-cells (4, 5). While patients with high $CD8^+$ T cell density in the tumor infiltrate have a more favorable prognosis across many other neoplastic diseases (6), little is still known about the presence and clinical impact of $CD8^+$ T cells in LCH-lesions (7–9).

Naive ($CD8^+$) T cells require antigen binding by their T cell receptor and co-stimulatory signals for (proper) activation. Previous studies have already demonstrated that LCH-cells express the co-stimulatory receptors CD40 (10–12), CD80 (3, 11–13), ICOS ligand (ICOSL) (14) and, although variably, CD86 (3, 11, 12) *in situ*. Moreover, transcriptome analyses revealed that LCH-cells express similar levels of CD40, CD80, and CD86 messenger RNA when compared to normal epidermal $CD207^+$ Langerhans cells (15, 16), and that they confer high expression of genes relevant for antigen presentation (including *CD1E*) and genes encoding members of the HLA (class II) complex (17). Thus, LCH-cells do not appear to have an intrinsic defect in their capacity to elicit a T cell immune response (12). This may explain why a proportion of LCH-lesional T cells have been shown to express cell surface markers indicative of recent activation (2), including CD40L (10), ICOS (14), CXCR3 (7), CD25 (5, 14), PD-1 (18, 19), RANKL (20), and CD45RO (7). In addition, marked monoclonal expansion of LCH-lesion infiltrating $CD3^+$ T cells has been observed (19), suggesting that T cell receptor activation occurred *in situ*. The antigen-specificity of activated LCH-lesional T cells is, however, unknown (2).

In 2010, universal activation of the mitogen-activated protein kinase (MAPK) signaling pathway in LCH-cells was demonstrated (21, 22). Since then, recurrent somatic mutations in genes of the MAPK signaling pathway have been identified in ∼85% of LCH-patients (23, 24). Oncogenic driver mutations are essential for tumorigenesis and tend to be clonally conserved. This makes neoantigens derived from proteins encoded by oncogenes highly attractive targets for immunotherapy. In addition, the natural T cell pool should contain T cells expressing high affinity T cell receptors for these neoantigens (25), which may exert potent antitumor function (26–29). This requires, however, neoantigens to be stably presented by Human Leukocyte Antigen (HLA) class I molecules and sufficient numbers of $CD8^+$ T cells at tumor sites. Over the past years, several HLA class I presented "public" neoantigens resulting from recurrent hotspot mutations in driver oncogenes have been discovered (30–38). Approximately 50–60% of LCH-patients carry the somatic $BRAF^{V600E}$ driver mutation (1, 21). $CD8^+$ T cells specific for $BRAF^{V600E}$ derived neopeptides have already been reported *in vitro* and in murine models (39–42). Thus, activation of LCH-lesional $BRAF^{V600E}$ neoantigen-specific $CD8^+$ T cells could hypothetically lead to the eradication of $BRAF^{V600E}$ expressing LCH-cells. Moreover, the concurrent formation of long-lasting bone-marrow homing memory $CD8^+$ T cells could control new outgrowth of residual $BRAF^{V600E}$ mutated histiocyte precursor cells (43). Immunotherapy specifically aimed at enhancing the number and effector function of these $BRAF^{V600E}$-specific $CD8^+$ T cells could offer great promise in the treatment of high-risk LCH-patients, given that these patients often bear the $BRAF^{V600E}$ mutation and fail first-line chemotherapy (44). Importantly, the *BRAF* gene is mutated in ∼7% of human cancers, with the $BRAF^{V600E}$ mutation accounting for >90% of all genetic variations (45, 46). Hence, the identification of HLA class I presented "public" neoantigens derived from the $BRAF^{V600E}$ protein would offer great therapeutic opportunity for many patients with other $BRAF^{V600E}$ mutated neoplasms as well (47).

The aim of this study was therefore to (i) assess the presence and clinical impact of lesional $CD8^+$ T cells in (HLA and $BRAF^{V600E}$) genotyped LCH-patients, and (ii) to investigate whether $BRAF^{V600E}$ derived neopeptides are presented by HLA class I molecules and could be recognized by such $CD8^+$ T cells.

MATERIALS AND METHODS

Patients and Samples

Patient accrual started after approval of the study protocol (CCMO NL33428.058.10) by each local Institutional Review Board. Only patients of whom formalin-fixed-paraffin-embedded (FFPE) first disease onset (FDO) LCH tissue biopsies were available were asked to participate in the study. Informed consent was provided by $n = 135$ patients and/or their parents/legal guardians. LCH diagnosis was confirmed by a combination of clinical findings and the presence of phenotypically aberrant $CD1a^+$ histiocytes in the tissue biopsy. The tissue samples were handled according to the code of conduct for proper secondary use of human tissue of the Federation of Dutch Medical Scientific Societies (FEDERA). Clinical information was collected by each participating center separately using a standardized Case Report Form (CRF) and anonymized data were provided to the researchers of the LUMC. Events were defined as LCH disease progression or reactivation. Progression was defined as (i) progression of existing lesions requiring start or intensification of systemic chemotherapy and/or radiotherapy, or (ii) the development of new lesions when Non-Active Disease (NAD) state had not yet been attained. LCH reactivation was defined as the development of new lesions after NAD had been attained for LCH FDO.

Flow Cytometric Analysis of LCH Tissue Biopsies

Fresh LCH tissue was dissociated using a gentle MACS tissue dissociator (Miltenyi Biotec) and single cells were cryopreserved in DMSO and albumin containing Roswell Park Memorial Institute (RPMI) culture medium. Before flow cytometric analysis, cells were thawed in RPMI + 20% fetal calf serum (FCS) + Penicillin-Streptomycin (P/S) containing 1,600 IU/ml DNAase (Sigma-Aldrich). After washing, the cells were stained with a mixture of different antibodies: CD45 (2D1, 1:50, BD Biosciences), CD1a (HI149, 1:50, BD Biosciences), CD207 (DCGM4, 1:25, Beckman Coulter), CD14 (MØP9, 1:20, BD Biosciences), CD3 (UCHT1, 1:200, BD Biosciences), CD8 (SK1, 1:100, BD Biosciences), HLA-DR (G46-6, 1:200, BD Biosciences), and panHLA class I (G46-2.6, 1:40, BD Biosciences). The cells were then re-washed and immediately analyzed on a FACS ARIA3 or FACS Fusion cell sorter (BD Biosciences).

HLA Genotyping and Analysis

High-resolution HLA genotyping was performed by DKMS Life Sciences Lab on DNA extracted from buccal swabs obtained from $n = 104$ LCH-patients using an ampliqon sequencing-based approach, as previously described (48, 49). For $n = 14$ additional patients, low-resolution HLA genotype data were acquired using a sequence specific oligoprimer-based approach (50). Hardy-Weinberg Equilibrium testing and HLA association analyses were performed using the HLA genotype data of Dutch LCH-patients. To evaluate statistical significance, two-sided Fisher's exact tests were carried out. The p-values were corrected for multiple comparisons conform the Šidák method (51). Odds ratios and corresponding 95% confidence intervals were calculated according to the method of Woolf with the Haldane correction (52, 53). Since a large control group could lead to significant differences that are clinically irrelevant, p-values were standardized to a smaller control sample size following the method of Good (54). The smaller control sample size was obtained using the following calculation: the total number of LCH-patients plus 3 times the number of patients as maximum allowed size for the control group.

Immunohistochemical Staining of LCH Tissue Sections

FFPE tissue sections (4–10 μm) were deposited on Superfrost™ (Thermo Fisher Scientific) glass slides, dried overnight at 37°C and stored at 4°C. Prior to immunohistochemical (IHC) staining, selected 4 μm slides were preheated at 66°C for 1 h and deparaffinized in xylol. For enzymatic CD1a IHC staining, endogenous peroxidase was blocked using Methanol/0.3% H_2O_2 for 20 min, before slides were rehydrated in ethanol and demineralized in water baths. Antigen retrieval was performed in boiling citrate buffer (pH 6.0) for 10 min and sections were incubated overnight with mouse IgG1-anti-human CD1a antibody (Clone 010, 1:800, DAKO) diluted in phosphate buffered saline (PBS)/0.5% bovine serum albumin (BSA). The next day, Envision+ System-HRP labeled polymer anti-mouse (DAKO) was applied for 30 min and color development was attained using commercial DAB+ (DAKO) for 10 min in the dark. This reaction was stopped using demineralized water and slides were counterstained with Mayer's hematoxylin (Klinipath) for 5 s prior to mounting with Pertex (Leica Microsystems).

An earlier published protocol was used for triple CD1a/CD3/CD8 fluorescent IHC staining (14). In brief, antigen retrieval was performed in boiling EDTA buffer (pH 8.0) for 10 min followed by a blocking step using 10% Normal Goat Serum in PBS/0.5% BSA for 15 min at room temperature. Slides were incubated overnight with the following primary antibody mix: rabbit IgG-anti-human CD3 (polyclonal, 1:300, DAKO), mouse IgG2b-anti-human CD8 (clone 4B11, 1:100, Novocastra, via Leica Microsystems), and mouse IgG1-anti-human CD1a (Clone 010, 1:400, DAKO). The next day, tissue slides were incubated for 30 min in the dark with 1:300 diluted goat-anti-mouse IgG1 Alexa Fluor 488, goat-anti-mouse IgG2b Alexa Fluor 546 and goat-anti-mouse IgG2a Alexa Fluor 647 antibodies (all from Invitrogen, via Thermofisher Life Technologies Europe). After washing in PBS, the sections were mounted with Mowiol (homemade) or Prolong Gold (Thermo Fisher Scientific) and stored in the dark at 4°C.

$BRAF^{V600E}$ Mutation Analysis

$CD1a^+$ enriched tissue parts were marked by a blinded pathologist on enzymatically CD1a stained LCH tissue slides.

Based on these reference slides, $CD1a^+$ enriched tissue parts were manually microdissected from multiple consecutively cut 10 µm tissue sections prepared from the remainder of the LCH tissue blocks. Total nucleic acid was automatically isolated from microdissected tissue using the Siemens Tissue Preparation System (Siemens Healthcare) robot (55). Presence of the $BRAF^{V600E}$ mutation was assessed by allele-specific real-time qPCR, as previously described (56). Of the $n = 54$ $BRAF^{V600E}$ negative samples, absence of the $BRAF^{V600E}$ mutation was confirmed in 46 samples (85%) by next-generation sequencing ($n = 39$), whole exome sequencing ($n = 1$) (57) or $BRAF^{V600E}$ droplet digital PCR ($n = 6$).

Quantification of T Cell Density in LCH-Lesions

For the manual cell counting method, multiple representative images were taken of each tissue slide at 400× magnification using a conventional Leica DM5500 fluorescent microscope equipped with LAS AF software (Leica Microsystems). Images were solely taken of representative areas containing phenotypically aberrant $CD1a^+$ LCH-cells. Using Image J software (version 1.47v) with the public Cell Counter plugin, fluorescently stained $CD1a^+$, $CD3^+CD8^-$ and $CD3^+CD8^+$ cells were manually counted in all images by two independent researchers (PGK and ECS) who were unaware of patient identity and outcome data. The cell counts of the individual images were added to form total $CD1a^+$, $CD3^+CD8^-$ and $CD3^+CD8^+$ cell counts. When total cell counts differed more than 10% between the two researchers, a third researcher (AGSH) reviewed the cell counting results and selected the most appropriate scoring (19/101 cases). Total $CD3^+$ cell counts were obtained by adding total $CD3^+CD8^-$ and $CD3^+CD8^+$ cell counts. To adjust for substantial differences in biopsy size between different patients, which may lead to profound disparities in absolute numbers of counted cells, ratios between the final numbers of total $CD3^+$ and $CD3^+CD8^+$ T cells and $CD1a^+$ LCH-cells were calculated for each patient.

For the manual semi-quantitative eyeball estimation method, whole slide images were taken of the same immunostained tissue slides at 400× magnification using a Pannoramic 250 Flash II slidescanner (3DHISTECH). These images were scored semi-quantitatively for LCH-lesional $CD3^+$ and $CD3^+CD8^+$ T cell density as has been previously described (58, 59): 1+, no, or sporadic T cells; 2+, moderate number of T cells; 3+, abundant occurrence of T cells; and 4+, highly abundant occurrence of T cells. Scoring examples are shown in **Figure S1**. Unfortunately, $n = 21/101$ (21%) of the tissue slides could not be reanalyzed due to considerable photobleaching of the fluorophores, induced by the earlier collection of high-power images for the manual cell counting analysis. Slides were scored independently by three researchers (PGK, ECS and AGSH). When scorings between two or more researchers differed more than 1 value (15/80 cases), the scoring was reviewed by all three researchers collectively and a consensus score was attained. Otherwise, the average score of the three scorings determined the final result, rounded to the nearest whole value (1–4+).

Whole slide images of sufficient quality (without significant color casts and/or folded tissue parts that are highly autofluorescent and/or out of focus) from $n = 48$ LCH-patients were analyzed using a quantitative automated digital image analysis method (**Figure S2**). First, the LCH-lesion and its directly adjacent T cells were encircled in the whole slide image in CaseViewer software and exported. In this way, cells that clearly did not belong to the microenvironment of the $CD1a^+$ LCH-cells were excluded. Using a custom in-house developed macro in ImageJ software, a white balance was then set for each individual exported image by designating background, foreground and autofluorescence. Next, uniform color thresholds for green ($CD1a^+$), red ($CD3^+CD8^-$), and purple ($CD3^+CD8^+$) were applied to all images, so that only green, red, and purple areas with color intensities higher than the threshold remained. Since automated quantification of individual cells was not feasible, the cumulative area of the remaining green, red, and purple areas was measured for each image, representing the total quantity of $CD1a^+$, $CD3^+CD8^-$, and $CD3^+CD8^+$ cells. Purple and Red ($CD3^+$) area/Green ($CD1a^+$) area and Purple ($CD3^+CD8^+$) area/Green ($CD1a^+$) area ratios could then be calculated for each patient. Comparison of the results obtained using our three separate analysis methods showed substantial concordance (**Figure S3**), supporting the validity of the findings in this study.

In vitro Peptide-HLA Class I Binding Analysis

Competition-based peptide-HLA class I binding assays were performed as previously described (60). The HLA binding affinities of the target peptides and strong binding reference peptides are expressed as the concentration that inhibits 50% binding of a fluorescently-labeled standard peptide (IC_{50}). The standard peptides were FLPSDCFPSV for HLA-A*02:01 and KVFPCALINK for HLA-A*03:01 and HLA-A*11:01. Notably, the ratio between the IC_{50} of a target peptide and the IC_{50} of an established strong binding reference peptide (for example 260:250 vs. 100:5) provides superior information on the true HLA class I binding capacity of the target peptide than the absolute IC_{50} of the target peptide.

Generation of $BRAF^{V600E}$ Expressing EBV-LCLs

The full length $BRAF^{V600E}$ sequence incorporated in a pBABE-Puro-BRAF-V600E plasmid was re-cloned into a LZRS-ires-Green Fluorescent Protein (GFP) retroviral vector by introducing the SwaI restriction site and a kozak sequence in front of the ATG start codon at the 5′end of the $BRAF^{V600E}$ sequence using Phusio DNA polymerase. In addition, a stop codon and NotI restriction site was introduced at the 3′end of the $BRAF^{V600E}$ sequence. The original pBABE-Puro-BRAF-V600E plasmid was kindly provided by William Hahn (Addgene plasmid #15269; http://n2t.net/addgene:15269; RRID:Addgene_15269) (61). Ligation of the *BRAF* PCR product in the LZRS vector digested with SwaI and NotI was performed overnight at 16°C. Prior to spin inoculation of Phoenix packaging cells, the correct sequence of the re-cloned $BRAF^{V600E}$ gene was confirmed by

Sanger sequencing (data not shown). Retrovirus containing supernatant was subsequently used to transduce Epstein-Barr virus-immortalized B cell lines (EBV-LCLs) with either a control empty LZRS vector (mock transduced EBV-LCL) or with the new *BRAF*^{V600E} containing LZRS vector (*BRAF*^{V600E} transduced EBV-LCL). Stably transduced GFP^{high} cells were purified using an ARIA3 flow cytometer prior to bulk expansion in RPMI medium containing 10% bovine serum.

Mass Spectrometry-Based Targeted Peptidomics

Cells were lysed at a concentration of 100e6 cells/ml lysis buffer [50 mM Tris-Cl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Zwittergent 3–12 (N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate) and protease inhibitor (Complete, Roche Applied Science)] for 2 h at 0°C (62). Lysates were successively centrifuged for 10 min at 2,500 × g and for 45 min at 31,000 × g to remove nuclei and other insoluble material, respectively. Next, lysates were cleared through a CL-4B Sepharose column (1 ml/1e9 cells) and passed through an anti-panHLA class I column containing 2.5 mg W6/32 IgG per ml protein A Sepharose (62). The W6/32 column was washed three times each with 1 ml of lysis buffer, 3 ml of low salt buffer (20 mM Tris-Cl pH 8.0, 120 mM NaCl), 1 ml of high salt buffer (20 mM Tris-Cl pH 8.0, 1 M NaCl), and finally with 3 ml of low salt buffer. Peptides were eluted with 5 ml of 10% acetic acid per ml column, diluted with 10 ml of 0.1% formic acid and purified by SPE (Oasis HLB, Waters) using 20 and 30% acetonitrile in 0.1% formic acid to elute the peptides.

For parallel reaction monitoring (PRM) analyses, the samples were lyophilized and resuspended in buffer A. HLA-eluates were injected together with a mix of 40 fmol of each heavy labeled peptide. The Orbitrap Fusion LUMOS mass spectrometer was operated in PRM-mode. Peptides KIGDFGLAT**E**, KIGDFGLAT**V**, KIGDFGLAT**E**K, and KIGDFGLAT**V**K were monitored. Selected peptides, the transitions and collision energies can be found in **Table S1**. The isolation width of Q1 was 1.2 Da. MS2 resolution was 35,000 at an AGC target value of 1 million at a maximum fill time of 100 ms. The gradient was run from 2 to 36% solvent B (20/80/0.1 water/acetonitrile/formic acid (FA) v/v) in 120 min. The nano-HPLC column was drawn to a tip of ∼5 μm and acted as the electrospray needle of the MS source. PRM data analysis and data integration were performed in Skyline 3.6.0.10493. Peptide abundances were calculated by comparing the peak area of the eluted (light) and the peak area of the spiked-in heavy peptides.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 8.0.1 and IBM SPSS Statistics version 25. Comparisons of (sub)groups were performed with the Mann-Whitney U test for continuous data and the Fisher exact test for categorical data. The Cox proportional hazards model was used for univariate analysis. Notably, log transformation of the widely differing CD8$^+$ T cell:CD1a$^+$ LCH-cell ratios was performed to increase the validity of the univariate analysis. Survival curves were estimated with the Kaplan-Meier method and compared with the Log-rank test. A p-value of <0.05 was considered statistically significant.

RESULTS

LCH-Cells Express Normal Levels of HLA Class I and II Molecules at Their Cell Surface

Since loss or downregulation of HLA expression has been shown to be a major tumor escape mechanism from T lymphocytes in a wide variety of cancers (63), we first evaluated by flow cytometric analysis the levels of HLA class I and HLA-DR expression on the surface of CD1a$^+$ (LCH-)cells present in $n = 6$ LCH-biopsies. The gating strategy applied is shown in **Figure S4**. The mean fluorescent intensity (MFI) of HLA class I and HLA-DR expression by CD1a$^+$ (LCH-)cells was comparable to MFI levels of HLA class I and HLA-DR expression by CD1a$^-$ CD14$^+$ (monocytic) cells present in the same LCH-biopsies (**Figure 1**; HLA class I, $p = 0.69$; HLA-DR, $p = 0.94$).

The HLA Genotype of LCH-Patients Does Not Differ From Healthy Controls

Besides HLA expression, HLA subtype is a crucial factor influencing whether a (neo)antigen is actually presented at the surface of nucleated cells. Several earlier published studies have suggested associations between particular HLA subtypes and LCH disease (extension) (64–67). To investigate this, we compared HLA genotype data from $n = 94$ Dutch LCH-patients to the HLA genotypes of 5,604 healthy Dutch blood donors reflecting the HLA genotype of the Dutch population (50). To maintain sufficient statistical power, HLA genotype was compared at low resolution level. No significant differences between Dutch LCH-patients and the Dutch reference population were observed (**Table S2**). Thus, our data do not support previous reports describing excess frequency of HLA-Bw61 and HLA-Cw7 (64), HLA-B7 and HLA-DR2 (65), and HLA-DR4 and/or HLA-Cw7 (66) genotypes in LCH-patients (**Tables S2, S3**). Moreover, our results neither confirm that LCH-patients with unifocal bone disease have significantly more often HLA-DR4 and/or HLA-Cw7 (66) subtypes nor that patients with single-system LCH have an increased prevalence of HLA-DRB1*03 (67) when compared to patients with multisystem LCH (**Table S4** and **Figure S5**, respectively).

BRAF^{V600E} Mutation Correlates With Decreased CD8$^+$ T Cell Density in LCH-Lesions

Assured that LCH-cells express HLA class I (and II) molecules and that there is a normal HLA subtype distribution among LCH-patients, we next investigated the presence of CD8$^+$ T cells in LCH-lesions. Various methods for the quantification of cell numbers in (specific areas of) tissue sections exist, including eyeball estimation, manual cell counting and automated digital image analysis. Although automated digital image analysis is increasingly being applied, manual cell counting is still considered the golden standard (68). Accordingly, we first determined the relative number of total CD3$^+$ and CD3$^+$CD8$^+$

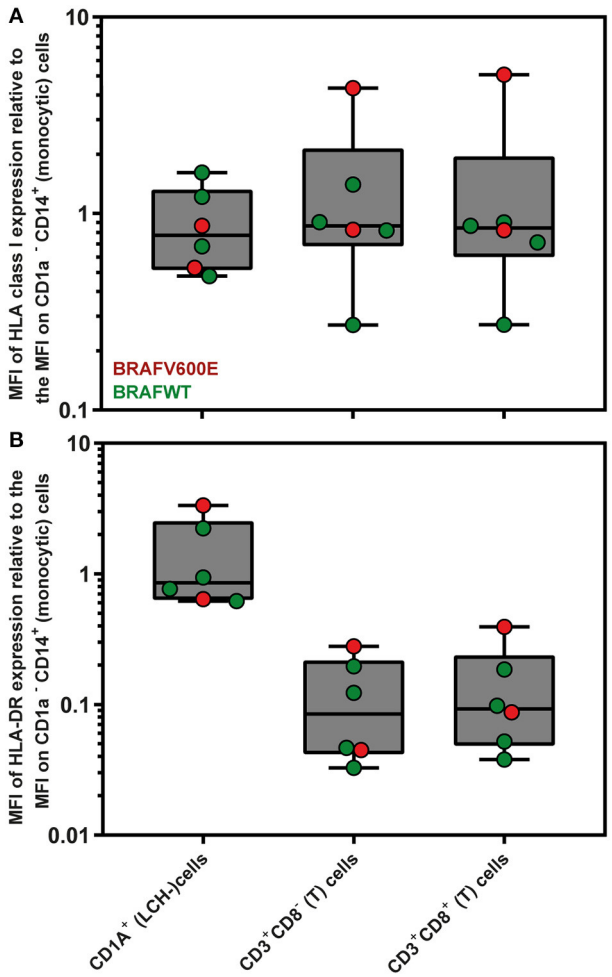

FIGURE 1 | Flow cytometric measurements of HLA class I and HLA-DR expression by LCH biopsy-derived hematopoietic cells. Mean Fluorescent Intensity (MFI) of **(A)** HLA class I (W6/32) and **(B)** HLA-DR expression by live CD1a$^+$ (LCH-)cells, CD3$^+$CD8$^-$ (T) cells and CD3$^+$CD8$^+$ (T) cells are depicted relative to the MFI of HLA class I or HLA-DR expression by CD1a$^-$ CD14$^+$ (monocytic) cells. The gating strategy applied is shown in **Figure S4**. The box extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The whiskers go down to the smallest value and up to the largest. Each individual value is plotted as a point superimposed on the graph.

T cells in LCH-lesions using this method. Fluorescently stained CD1a$^+$, CD3$^+$CD8$^-$ and CD3$^+$CD8$^+$ cells (**Figure 2A**) were manually counted in LCH-biopsies from $n = 101$ patients collected at first disease onset using the public ImageJ Cell Counter plugin. A median of 1,810 cells (range: 188–9,301) were counted in a median of 16 representative images (range: 2–56) taken at 400× magnification of tissue areas containing phenotypically aberrant CD1a$^+$ LCH-cells. Large inter- and intrapatient heterogeneity was seen in the relative number of LCH-lesional CD3$^+$ and CD8$^+$ T lymphocytes (**Figure S6** and **Figure 2B**, respectively). Calculated CD8$^+$ T cell:CD1a$^+$ LCH-cell ratios (CD8 ratios) ranged from 0.00 to 4.96. The median CD8 ratio was 0.06, corresponding to 1 CD8$^+$ T cell per 16 CD1a$^+$ LCH-cells. No significant difference in LCH-lesional CD8 ratios was observed between bone and skin biopsies ($p = 0.37$) nor between patients with single- or multisystem LCH disease ($p = 0.55$). Yet, *BRAF*^{V600E} mutated patients displayed significantly lower LCH-lesional CD8 ratios when compared to *BRAF* wildtype (*BRAF*^{WT}) patients ($p = 0.01$; **Figure 2C**). *BRAF*^{V600E} mutated LCH-lesions had a median CD8 ratio of 0.0316, corresponding to 1 CD8$^+$ T cell per 32 CD1a$^+$ LCH-cells. In contrast, *BRAF*^{WT} lesions had a median CD8 ratio of 0.0775, corresponding to 1 CD8$^+$ T cell per 13 CD1a$^+$ LCH-cells. *BRAF*^{V600E} mutated lesions also had significantly lower total CD3$^+$ T cell:CD1a$^+$ LCH-cell ratios than *BRAF*^{WT} lesions ($p = 0.001$; **Figure S7**). As manual selection of representative tissue areas may introduce bias, we also analyzed whole slide images taken from a subset of immunostained tissue sections using a previously described semi-quantitative eyeball estimation method (58, 59) (**Figure S1**) and a quantitative automated digital image analysis method (**Figure S2**). The correlation between the *BRAF*^{V600E} mutation and decreased LCH-lesional CD3$^+$ and CD8$^+$ T cell density was confirmed by these two additional analysis methods (**Figure S8**).

Lesional CD8$^+$ T Cell Density Does Not Correlate With Event-Free Survival in LCH

We subsequently assessed whether lesional CD8$^+$ T cell density is of prognostic value in LCH. Using univariate cox regression analysis, no significant association was observed between LCH-lesional CD8 ratio and event-free survival ($p = 0.46$; Hazard Ratio= 0.89; 95% Confidence Interval = 0.66–1.21). In addition, no significant difference was present when patients were divided by a median split, grouped in patients with HIGH or LOW CD8 ratios (**Figure 2B** and **Table 1**) and compared with regard to event-free survival ($p = 0.96$, **Figure 2D**). Thus, LCH-lesional CD8$^+$ T cell density did not correlate with disease outcome in this retrospective patient cohort.

The *BRAF*^{V600E} Derived Neopeptide KIGDFGLATEK Binds to HLA-A*03:01 and HLA-A*11:01

To investigate the immunogenicity of the *BRAF*^{V600E} mutation, we used the online NetMHC 4.0 server (69) to explore putative HLA class I binding 8–12 amino acid long (8–12mer) neopeptides derived from the *BRAF*^{V600E} protein. In addition, NetCHOP 3.1 software (70) was used to predict proteasomal cleavage motifs and thereby identify peptides that are presumably generated by the human proteasome. From all 8–12mer *BRAF*^{V600E} derived neopeptides that are generated by the human proteasome according to NetCHOP, only a single neopeptide, the 11mer KIGDFGLATEK, is predicted to bind to one or more of the analyzed HLA class I molecules (**Table S5**). According to NetMHC, KIGDFGLATEK binds weakly to HLA-A*11:01 and HLA-A*03:01, expressed by respectively $n = 11/104$ (11%) and $n = 25/104$ (24%) LCH-patients from our cohort. The remainder of the 8–12mer *BRAF*^{V600E} derived neopeptides are all considered not be

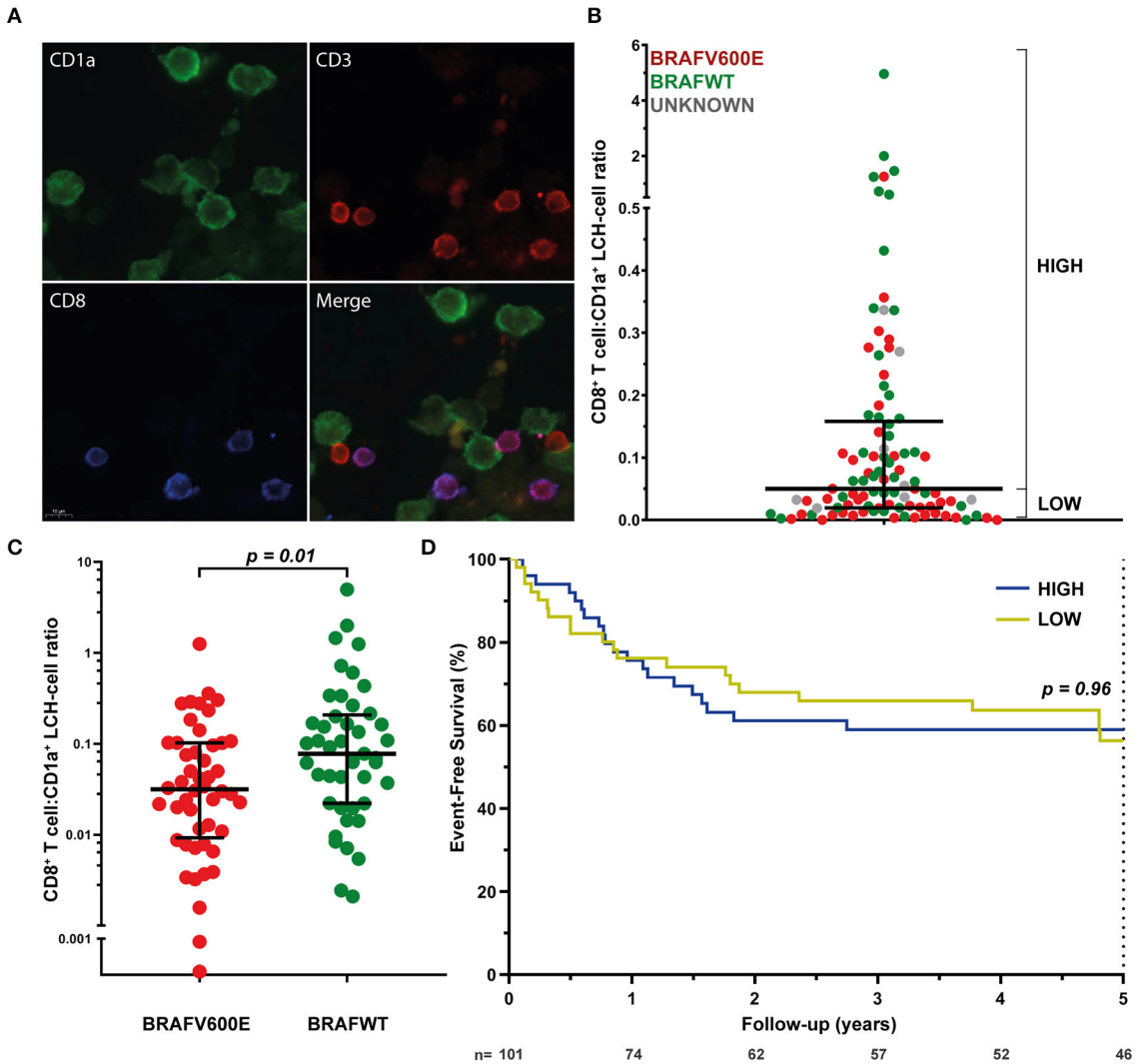

FIGURE 2 | LCH-lesional CD8$^+$ T cell densities in first disease onset tissue biopsies of LCH-patients. **(A)** Representative images of immunostained phenotypically aberrant CD1a$^+$ LCH-cells (green), CD3$^+$CD8$^-$ T cells (red), and CD3$^+$CD8$^+$ T cells (purple) that were manually counted. **(B)** Distribution of LCH-lesional CD8$^+$ T cell:CD1a$^+$ LCH-cell ratios (CD8 ratios) in first-disease onset tissue biopsies of $n = 101$ LCH-patients. For Kaplan-Meier survival analysis (shown in **D**), patients were divided by a median split and grouped in patients with HIGH or LOW CD8 ratios. **(C)** Distribution of CD8 ratios in *BRAF*^{V600E} mutated ($n = 48$) and *BRAF* wildtype ($n = 45$) LCH-lesions. **(D)** Kaplan-Meier survival analysis of patients with HIGH ($n = 50$) or LOW ($n = 51$) CD8 ratios. Event was defined as LCH disease progression or reactivation. N, number of patients at risk.

generated by the human proteasome and/or to be non-binders. Additional *in vitro* peptide-HLA binding studies however demonstrated that KIGDFGLATEK binds with comparable affinity to HLA-A*11:01 as the strong binding reference peptide (QVPLRPMTYK) that was used in our competition-based peptide-HLA binding assay (60). In line with the predicted binding affinity, this neopeptide was shown to also bind, albeit less efficiently, to HLA-A*03:01 (**Table 2**), as evidenced by the small difference in nanomolar concentration that inhibited 50% binding (IC_{50}) of the fluorescently-labeled standard peptide (KVFPCALINK) between KIGDFGLATEK and QVPLRPMTYK (672 vs. 297 nM, respectively). Notably, NetMHCstab 1.0 software (71) predicts that the KIGDFGLATEK-HLA-A*11:01 complex is highly stable (predicted half-life: 8.87 h) and that the KIGDFGLATEK-HLA-A*03:01 complex is weakly stable (predicted half-life: 3.29 h). We also assessed the *in vitro* HLA binding affinity of the 11mer KIGDFGLATVK and 10mer KIGDFGLATV *BRAF* wildtype peptides and of the 10mer KIGDFGLATE neopeptide (**Table 2**). In accordance with the predictions made by NetMHC, KIGDFGLATVK was shown to bind with comparable affinity to HLA-A*11:01 as the strong binding reference peptide QVPLRPMTYK, and to confer weaker binding to HLA-A*03:01, just like KIGDFGLATEK. Moreover, the 10mer *BRAF* wildtype peptide KIGDFGLATV was shown to bind with comparable affinity to HLA-A*02:01 as the strong binding reference peptide

TABLE 1 | Characteristics of LCH-patients from whom biopsies were analyzed for LCH-lesional $CD8^+$ T cell density.

	All patients	High LCH-lesional CD8 ratio	Low LCH-lesional CD8 ratio	*P*-value
Patients	101	50 (50%)	51 (50%)	
Gender				
Male	53 (52%)	29 (58%)	24 (47%)	0.32
Female	48 (48%)	21 (42%)	27 (53%)	
Age distribution				
Pediatric patients	85 (84%)	40 (80%)	45 (88%)	0.29
Adult patients	16 (16%)	10 (10%)	6 (12%)	
Disease extension				
SS	78 (77%)	39 (78%)	39 (76%)	1
MS RO–	12 (12%)	5 (10%)	7 (14%)	0.76
MS RO+	11 (11%)	6 (12%)	5 (10%)	0.76
Mutation status				
$BRAF^{V600E}$ positive	48 (48%)	18 (36%)	30 (59%)	0.02
$BRAF^{V600E}$ negative	45 (45%)	28 (56%)	17 (33%)	
Unknown	8 (8%)	4 (8%)	4 (8%)	
Chemotherapy for FDO	34 (34%)	15 (30%)	19 (37%)	0.53
Follow-up (median)	10.1 years	8.5 years	11.3 years	0.36

SS, single-system LCH disease; MS RO–, multisystem LCH disease without risk organ (bone marrow, liver and/or spleen) involvement; MS RO+, multisystem LCH disease with risk organ involvement; FDO, first disease onset; CD8 ratio, $CD8^+$ T cell:$CD1a^+$ LCH-cell ratio.

(FLPSDFFPSV) used in our assay. In contrast, its mutant counterpart KIGDFGLATE does not bind at all to this particular HLA class I molecule.

HLA-A*11:01 and/or HLA-A*03:01 Genotype Is Not Associated With Increased Event-Free Survival in $BRAF^{V600E}$ Mutated LCH-Patients

Having established that the $BRAF^{V600E}$ derived neopeptide KIGDFGLATEK can bind to two HLA class I molecules that are relatively frequent in the Caucasian population, we evaluated whether $BRAF^{V600E}$ mutated LCH-patients expressing HLA-A*03:01 and/or HLA-A*11:01 had increased event-free survival as compared to LCH-patients without these HLA genotypes. High-resolution HLA genotype data was available for $n = 48$ $BRAF^{V600E}$ mutated LCH-patients. Patient characteristics are shown in **Table S6**. No significant difference in event-free survival was observed between $BRAF^{V600E}$ mutated LCH-patients with and without HLA-A*03:01 and/or HLA-A*11:01 ($p = 0.32$, **Figure S9**).

KIGDFGLATEK Is Not Detected in the HLA Class I Peptidome of $BRAF^{V600E}$ Expressing Cells

To assess whether KIGDFGLATEK is actually presented on the surface of cells that express $BRAF^{V600E}$ and HLA-A*03:01 and/or HLA-A*11:01, we performed mass spectrometry-based targeted peptidomics of HLA class I presented peptides isolated from various EBV-LCL transduced with a LZRS-retroviral vector containing full length $BRAF^{V600E}$ protein and reporter Green Fluorescent Protein (GFP) encoding DNA sequences. Based on the results of the *in silico* analysis and *in vitro* peptide-HLA binding assays, three different EBV-LCL were selected for the transduction experiments with HLA-A*03:01/HLA-A*02:01 (SB), HLA-A*11:01/HLA-A*02:01 (MLA), and HLA-A*02:01/HLA-A*02:01 (JY) genotypes. Extended HLA genotypes are shown in **Table S7**. After retroviral transduction, GFP^{high} cells were sorted and expanded in bulk. JY and MLA cell lines that were mock transduced with a control (empty-)GFP retroviral vector were analyzed in parallel. Flow cytometric analysis demonstrated that neither retroviral transduction with the $BRAF^{V600E}$ containing vector (**Figure S10**) nor transduction with the control empty vector (data not shown) altered HLA class I (W6/32) and HLA-DR expression at the cell surface. Moreover, HLA subtype-specific antibodies (kindly provided by Dr. D.L. Roelen, HLA genotyping laboratory LUMC, Leiden) confirmed normal HLA subtype expression by $BRAF^{V600E}$ transduced SB, MLA (**Figure S10**) and JY cells (data not shown). We also included an HLA-A*01/HLA-A*24 bearing $BRAF^{V600E}$ mutated colon carcinoma cell line (HT29) with earlier confirmed HLA (72–74) and $BRAF^{V600E}$ protein (75, 76) expression in our analysis. Using parallel reaction monitoring (PRM)-based targeted peptidomics (77), the 11mer neopeptide KIGDFGLATEK was not detected in the HLA class I peptidomes of both $BRAF^{V600E}$ expressing cell lines expressing HLA-A*03:01 or HLA-A*11:01 (**Table 3**). Notably, neither the 11mer *BRAF* wildtype peptide KIGDFGLATVK was detected in HLA class I peptides isolated from the mock or $BRAF^{V600E}$ transduced SB and MLA EBV-LCL. In contrast, the 10mer BRAF wildtype peptide KIGDFGLATV was detected in the HLA class I peptidomes of 3/3 $BRAF^{V600E}$ transduced and 2/2 mock transduced cell lines expressing HLA-A*02:01 (**Table 3**).

DISCUSSION

A large number of studies have demonstrated a positive association between overall $CD8^+$ T cell density in the tumor infiltrate and a favorable clinical prognosis in many different types of cancers (6). In this study, we did not observe such an association in a substantial cohort of LCH-patients with well-documented clinical outcome. This dissimilarity between LCH and other neoplastic disorders may be due to their vast differences in mutational load and, correspondingly, the number of T cell activating neoantigens that can arise from this mutational burden. Furthermore, the immune suppressive microenvironment in LCH-lesions (5, 14, 15, 18, 78–81) may hamper $CD8^+$ T cell infiltration (non-mutated), antigen recognition and cytolytic function .

TABLE 2 | *In silico* and *in vitro* HLA class I binding affinities of *BRAF*^{V600E} and *BRAF* wildtype protein-derived peptides and two strong binding reference peptides.

Peptide	Predicted proteasomal cleavage	Predicted HLA binding affinity (IC_{50}, nM)						*In vitro* HLA binding affinity (IC_{50}, nM)			500 nM
		NetMHC 3.4			NetMHC 4.0			Peptide-HLA binding assay			
	NetCHOP 3.1	A*02:01	A*03:01	A*11:01	A*02:01	A*03:01	A*11:01	A*02:01	A*03:01	A*11:01	
KIGDFGLATV	YES	38	17,310	23,823	107	13,515	19,725	40	NT	NT	**50 nM**
KIGDFGLATE	NO	15,997	20,112	23,609	19,485	22,113	23,743	86918	NT	NT	
KIGDFGLATVK	YES	25,719	448	163	3,347	191	363	NT	415	32	
KIGDFGLATEK	YES	23,298	622	98	29,947	278	345	NT	672	45	
QVPLRPMTYK	NR	31,545	77	62	32,523	21	37	NT	297	36	
FLPSDFFPSV	NR	4	24,267	27,281	4	19,261	22,553	8	NT	NT	**0 nM**

IC_{50}, the concentration that inhibits 50% binding of a fluorescently-labeled standard peptide; nM, nanomolar; NR, not relevant, because these are the strong binding reference peptides; NT, not tested. The color values correspond to the IC_{50} values 0–500 nM (0 dark red, 500 white).

TABLE 3 | Peptides detected using mass-spectrometry based targeted peptidomics of HLA class I peptides isolated from multiple *BRAF* wildtype or *BRAF*^{V600E} expressing cell lines.

Peptide	Cell line											
	JY mock		JY BRAF^{V600E}		MLA mock		MLA BRAF^{V600E}		SB BRAF^{V600E}		HT29	
	1,900 × 10e6*		51 × 10e6		158 × 10e6		170 × 10e6		28 × 10e6		1,800 × 10e6	
	A*02:01	A*02:01	A*02:01	A*02:01	A*02:01	A*11:01	A*02:01	A*11:01	A*02:01	A*03:01	A*01	A*24
KIGDFGLATV	+		+		+		+		+		–	
KIGDFGLATE	–		–		–		–		–		–	
KIGDFGLATVK	–		–		–		–		–		–	
KIGDFGLATEK	–		–		–		–		–		–	

*Mock, transduced with a control (empty-) GFP retroviral vector; BRAF^{V600E}, transduced with a BRAF^{V600E}-GFP retroviral vector; HT29, colon carcinoma cell line harboring the heterozygous BRAF^{V600E} mutation; +, peptide detected; –, peptide not detected; *, number of cells analyzed.*

In line with an earlier undetailed observation (7), the relative number of LCH-lesional CD8$^+$ T cells appears low in this study. Moreover, we demonstrate with three separate analysis methods that *BRAF*^{V600E} mutated LCH-patients display lower lesional CD3$^+$ and CD8$^+$ T cell densities than *BRAF* wildtype patients. Although the clinical significance of this latter observation is not immediately apparent, it does point out that the different MAPK pathway mutations expressed by neoplastic LCH-cells seem to have a distinct impact on their immune microenvironment. A number of studies on *BRAF*^{V600E} positive melanoma have already suggested that the *BRAF*^{V600E} mutation promotes immune evasion by upregulating the transcription of many immunomodulatory chemokine and cytokine genes as well as the internalization of cell surface HLA class I molecules (82, 83). The presence of many of these immunomodulatory chemokines and cytokines in LCH-lesions has been extensively demonstrated (2). Notably, we did however observe normal HLA class I expression by CD1a$^+$ (LCH-)cells in two *BRAF*^{V600E} positive LCH-biopsies analyzed by flow cytometry (**Figure 1A**), and showed that transduction of EBV-immortalized B cells with a *BRAF*^{V600E} encoding retroviral vector does not impair HLA class I expression. Zeng and colleagues recently described that *BRAF*^{V600E} mutated LCH-patients have significantly higher numbers of lesional Foxp3$^+$ regulatory T cells and increased PD-L1 expression by LCH-cells when compared to *BRAF*^{WT} patients (80). In accordance with this study, a preliminary report by Chakraborty and others also describes that *BRAF*^{V600E} expressing LCH-cells display higher expression levels of ligands for inhibitory receptors, including PD-L1/L2 and Galectin-9, when compared to *BRAF*^{WT} patients (19). Notably, the presence of PD-1 expressing T cells in LCH-lesions has been reported as well (18, 19), and was confirmed in (*BRAF*^{V600E} positive) patients from our cohort (**Figure S11**). PD-L1 blockade has been shown to induce expansion of tumor-infiltrating CD8$^+$ T cells (84). Thus, the reported increased PD-L1 expression by *BRAF*^{V600E} positive LCH-cells (19, 80) could explain the decreased LCH-lesional CD8$^+$ T cell density in *BRAF*^{V600E} mutated patients from our study. In addition, the immune suppressive microenvironment in LCH-lesions (5, 14, 15, 18, 78–81) may clarify why the rare CD8$^+$ T cells that did make it into these lesions had no significant clinical impact. This is supported by our own observation of low numbers of HLA-DR^{pos} LCH-lesional CD8$^+$ T cells (**Figure 1**), low numbers of "licensed-to-kill" CD8$^+$ T cells co-expressing the cytolytic enzymes Perforin and Granzyme B (85) (**Figure S12**), and rare presence of Caspase 3 expressing LCH-cells (data not shown). HLA-DR is widely recognized as a marker of T cell activation (86), and Caspase 3 is the hallmark marker of efficient target cell

apoptosis induced by activated CD8$^+$ T cells (87). In line with the recently reported defective response of LCH-lesion infiltrating T cells to allogeneic stimulation (19), these observations collectively suggest that CD8$^+$ T cells in LCH-lesions are often dysfunctional. Future studies using (imaging) mass cytometry, which allows the simultaneous detection of a multitude of cellular markers (with spatial context), are needed to study the phenotypic characteristics of LCH-lesional (CD8$^+$) T cells in more detail. Moreover, the alleged distinct impact of the different MAPK pathway mutations on the immune microenvironment of neoplastic LCH-cells should ideally be investigated in a LCH mouse model.

Encouraged by published results suggesting that *BRAF*^{V600E} protein-derived neopeptides can trigger antitumor immunity (41, 82), we used the most recent version of publicly accessible NetMHC software to explore putatively HLA class I binding neoantigens derived from the *BRAF*^{V600E} protein. Surprisingly, from all 8–12mer *BRAF*^{V600E} derived neopeptides that are predicted to be generated by the human proteasome by NetCHOP 3.1 software, only a single neopeptide (KIGDFGLAT**E**K) was predicted to bind to one or more of the analyzed HLA class I molecules. *In vitro* peptide-HLA binding assays confirmed the predicted binding capacity of KIGDFGLAT**E**K to HLA-A*03:01 and HLA-11*01. In contrast to the results generated with an earlier version of Syphpeiti software (41), the NetMHC 4.0 server did not qualify the two (putatively HLA-A*02:01 binding) neopeptides LAT**E**KSRWSG and LAT**E**KSRWS to be HLA-binders. Using PRM-based targeted peptidomics, KIGDFGLAT**E**K was not detected in the HLA class I peptidomes of 2/2 *BRAF*^{V600E} expressing EBV-LCL (MLA *BRAF*^{V600E} and SB *BRAF*^{V600E}) that expressed normal levels of HLA-A*03:01 or HLA-A*11:01. In contrast, the HLA-A*02:01 binding *BRAF* wildtype peptide KIGDFGLATV was traceable in HLA class I peptides isolated from 5/5 cell lines expressing this HLA subtype, verifying normal antigen processing in these cells and adequate sensitivity of our peptidomics approach. Since the 11mer *BRAF* wildtype peptide KIGDFGLATVK was not detected in mock (empty-GFP) nor *BRAF*^{V600E} transduced EBV-LCL as well, the apparent lack of KIGDFGLAT**E**K presentation at the cell surface seems not due to a competitive HLA binding disadvantage relative to its wildtype counterpart (88). Instead, both KIGDFGLAT**E**K and KIGDFGLATVK peptides may not be generated by the human proteasome. This could be explained by the fact that both HLA-A*03:01 and HLA-A*11:01 molecules exclusively bind peptides with lysine as the C-terminal anchor residue (89). NetCHOP software only produces neural network predictions for proteosomal cleavage. Protein cleavage yielding C-terminal lysine residues is, however, not readily accomplished by the human proteasomes alone. Instead, this process requires the cytosolic endopeptidases nardilysin and thimet oligopeptidase as well (89, 90). Another possibility is that the 11mer KIGDFGLATVK and KIGDFGLAT**E**K peptides are expressed at the cell surface, but that they are underrepresented among the large pool of naturally presented ligands eluted from peptide-HLA class I complexes, because of a common peptide length distribution including mostly 9mer peptides and far less 8mer, 10mer, and longer peptides (91). This is also demonstrated by the list of peptides that were detected using data-dependent acquisition-based peptidomics in the HLA class I peptide pools isolated from the mock transduced JY and MLA EBV-LCL (**Table S8**). The high sensitivity of targeted peptidomics makes this option however less probable, although it must be noted that lower numbers (28–170 × 10e6) of *BRAF*^{V600E} transduced SB, MLA, and JY B cells were subjected to analysis as compared to mock transduced B cells (158–1,900 × 10e6). This was because GFP^{high} *BRAF*^{V600E} transduced cells displayed intrinsically higher apoptosis rates leading to substantially lower yields (data not shown).

In addition to the importance of CD8$^+$ T cells, multiple studies have highlighted the importance of CD4$^+$ T cells in tumor rejection (34, 92–96). Notably, one study identified *BRAF*^{V600E}-specific CD4$^+$ T cells after repetitive peptide stimulation of peripheral blood mononuclear cells from three melanoma patients whose metastatic tumors harbored the *BRAF*^{V600E} mutation (40). Moreover, Veatch and colleagues recently identified HLA-DQB1*03-restricted *BRAF*^{V600E}-specific CD4$^+$ T cells in an acral melanoma patient, who nonetheless developed metastases under ipilimumab (anti-CTLA-4) immunotherapy (97). Unfortunately, the precise amino acid sequence of the recognized neoantigen was not reported. Available software tools to predict HLA class II binding peptides are known to be significantly less accurate than available algorithms for predicting HLA class I binding peptides. Moreover, the yield of *BRAF*^{V600E} transduced B cells expressing HLA-DQB1*03:02 (SB EBV-LCL) was far too small to elute sufficient quantities of peptide-HLA class II complexes needed for successful data-dependent acquisition-based peptidomics. We could, therefore, not confirm that this recently identified *BRAF*^{V600E} protein-derived HLA-DQB1*03 binding neopeptide is naturally processed and presented at the cell surface of our *BRAF*^{V600E} transduced HLA-DQB1*03 expressing EBV-LCL. We did however investigate whether *BRAF*^{V600E} mutated LCH-patients expressing HLA-DQB1*03 in general, or HLA-DQB1*03:02 and/or HLA-DQB1*03:03 in particular [due to their putative strongest binding and/or peptide-HLA complex stability (97)], had increased event-free survival when compared to *BRAF*^{V600E} mutated patients without these HLA genotypes. Notably, HLA class I subtype has already been demonstrated to influence response to checkpoint blockade immunotherapy in patients with diverse cancers (98). Neither *BRAF*^{V600E} mutated LCH-patients with HLA-DQB1*03 (n = 30, 62.5%) nor with HLA-DQB1*03:02 and/or HLA-DQB1*03:03 (n = 18, 37.5%) displayed increased event-free survival when compared to patients without these HLA alleles (p = 0.78 and p = 0.57, respectively; data not shown). Thus, although we agree that adoptive cell therapy with T cell receptor-engineered *BRAF*^{V600E}-specific CD4$^+$ T cells may offer great therapeutic potential, the clinical impact of potentially present *BRAF*^{V600E}-specific CD4$^+$ T cells in HLA-DQB1*03 bearing, *BRAF*^{V600E} mutated LCH-patients is questionable. Of note, the rare CD4$^+$ *BRAF*^{V600E}-specific T cells reported in the acral melanoma patient by Veatch et al. were not paralleled by *BRAF*^{V600E}-specific CD8$^+$ T cells, but by diverse CD8$^+$ T cells reactive to multiple melanoma-associated self-antigens. Whether such non-mutated tumor-associated antigens are also expressed by LCH-cells is of great interest and remains to be determined. This will however be challenging given the

(relatively) low numbers of neoplastic LCH-cells that can be obtained for peptidome analysis from fresh or frozen LCH tissue samples, which are in addition very scarce due to the rarity of the disease.

Since the generation of neoantigens is a probabilistic process (47), we can of course not rule out that other somatic mutations in LCH-cells are a source of neoantigens that are naturally processed and presented in (stable) peptide-HLA class I complexes. Based on recent insights, this probability is however very low. With the development of deep-sequencing technologies, comprehensive analyses of neoantigen-specific T cell responses have been carried out for a substantial number of cancer patients since 2013 (25, 26, 29). The striking conclusion that can now be drawn from these studies is that only a very small fraction of non-synonymous mutations leads to the formation of a neoantigen for which $CD4^+$ or $CD8^+$ T cell reactivity can be detected (25). Most melanomas and a sizable fraction of other high-prevalence cancers in adults have a mutational load above 10 somatic mutations per Mb, corresponding to ∼150 non-synonymous mutations within expressed genes (25, 99, 100). Even in melanoma patients, neoantigen-specific T cell reactivity is however not always observed (95). Thus, there is a growing awareness that tumor types with a mutational load below 10, and especially below 1 mutation(s) per Mb, are less likely to express neoantigens that can be recognized by autologous T cells (25). Although the total number of LCH samples analyzed by whole-exome sequencing (WES) is still small (101), a remarkably low frequency of somatic mutations in LCH-cells was found in the largest WES analysis to date ($n = 41$), with a median of 1 somatic mutation per patient (0.03 mutations per Mb) (22). Thus, the likelihood of neoantigen formation and concurrent induction of protective neoantigen-specific T cell responses in LCH-patients seems very low (25). Notably, Goyal and others recently demonstrated a low mutational burden in other histiocytic neoplasms as well (102). We therefore question the usefulness of classical immune checkpoint inhibitors for the treatment of relapsed or refractory LCH (or other histiocytic neoplasms), especially given that these LCH-patients often bear the $BRAF^{V600E}$ mutation (44), and that pretherapy intratumoral $CD8^+$ T cell density has been shown to positively correlate with mutational burden, neoantigen load and response to immune checkpoint inhibition in many other neoplastic diseases (103, 104).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethical Committee of the LUMC. Written informed consent to participate in this study was provided by the participants or by the participants' legal guardian.

AUTHOR CONTRIBUTIONS

PK, ES, AH, QA, JB, SV, AR, GJ, PV, GH, KF, RE, NS-W, and TW performed experiments and/or analyzed data. DR performed low-resolution HLA genotyping and provided HLA subtype-specific antibodies. LV helped with the automated digital image analysis. RV, CN, AC, and CH provided LCH tissue biopsies and marked $CD1a^+$ enriched tissue parts on CD1a stained slides that served as reference slides for the manual microdissection procedure. AB, JL, OA, and CB included patients and supervised the clinical data collection by PK, TZ, VL, and RME. AH, CB, and OA designed the study. PK and AH drafted the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.03045/full#supplementary-material

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REVIEW
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Neoantigens in Hematologic Malignancies

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T cell cancer neoantigens are created from peptides derived from cancer-specific aberrant proteins, such as mutated and fusion proteins, presented in complex with human leukocyte antigens on the cancer cell surface. Because expression of the aberrant target protein is exclusive to malignant cells, immunotherapy directed against neoantigens should avoid "on-target, off-tumor" toxicity. The efficacy of neoantigen vaccines in melanoma and glioblastoma and of adoptive transfer of neoantigen-specific T cells in epithelial tumors indicates that neoantigens are valid therapeutic targets. Improvements in sequencing technology and innovations in antigen discovery approaches have facilitated the identification of neoantigens. In comparison to many solid tumors, hematologic malignancies have few mutations and thus fewer potential neoantigens. Despite this, neoantigens have been identified in a wide variety of hematologic malignancies. These include mutated nucleophosmin1 and PML-RARA in acute myeloid leukemia, ETV6-RUNX1 fusions and other mutated proteins in acute lymphoblastic leukemia, BCR-ABL1 fusions in chronic myeloid leukemia, driver mutations in myeloproliferative neoplasms, immunoglobulins in lymphomas, and proteins derived from patient-specific mutations in chronic lymphoid leukemias. We will review advances in the field of neoantigen discovery, describe the spectrum of identified neoantigens in hematologic malignancies, and discuss the potential of these neoantigens for clinical translation.

Keywords: neoantigen, hematologic malignancies, human leukocyte antigen, T cell receptor, immunotherapy, mutations, fusion proteins

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INTRODUCTION

Neoantigens are composed of peptides derived from full-length aberrant cancer-specific proteins through a multi-step intracellular process that has been extensively reviewed (1–3) and presented in complex with human leukocyte antigen (HLA) molecules. This peptide-HLA complex is recognized by T cell receptors (TCRs). Non-viral neoantigens can be potentially be generated from any protein-coding mutations, fusion proteins, and cancer-specific splice isoforms (**Figure 1**), although not every aberrant protein will yield neoantigens. In the treatment of solid tumors, clinical successes have been seen with adoptive transfer of neoantigen-specific tumor-infiltrating lymphocytes (TIL) (4–7) and neoantigen vaccines (8–10), highlighting the importance of this class of antigens in effective anti-tumor immunity. T cell responses against neoantigens also appear to contribute to the efficacy of immune checkpoint blockade therapy (11, 12) and allogeneic hematopoietic cell transplantation (HCT) (13). Therapies targeting a neoantigen derived from an oncogenic driver

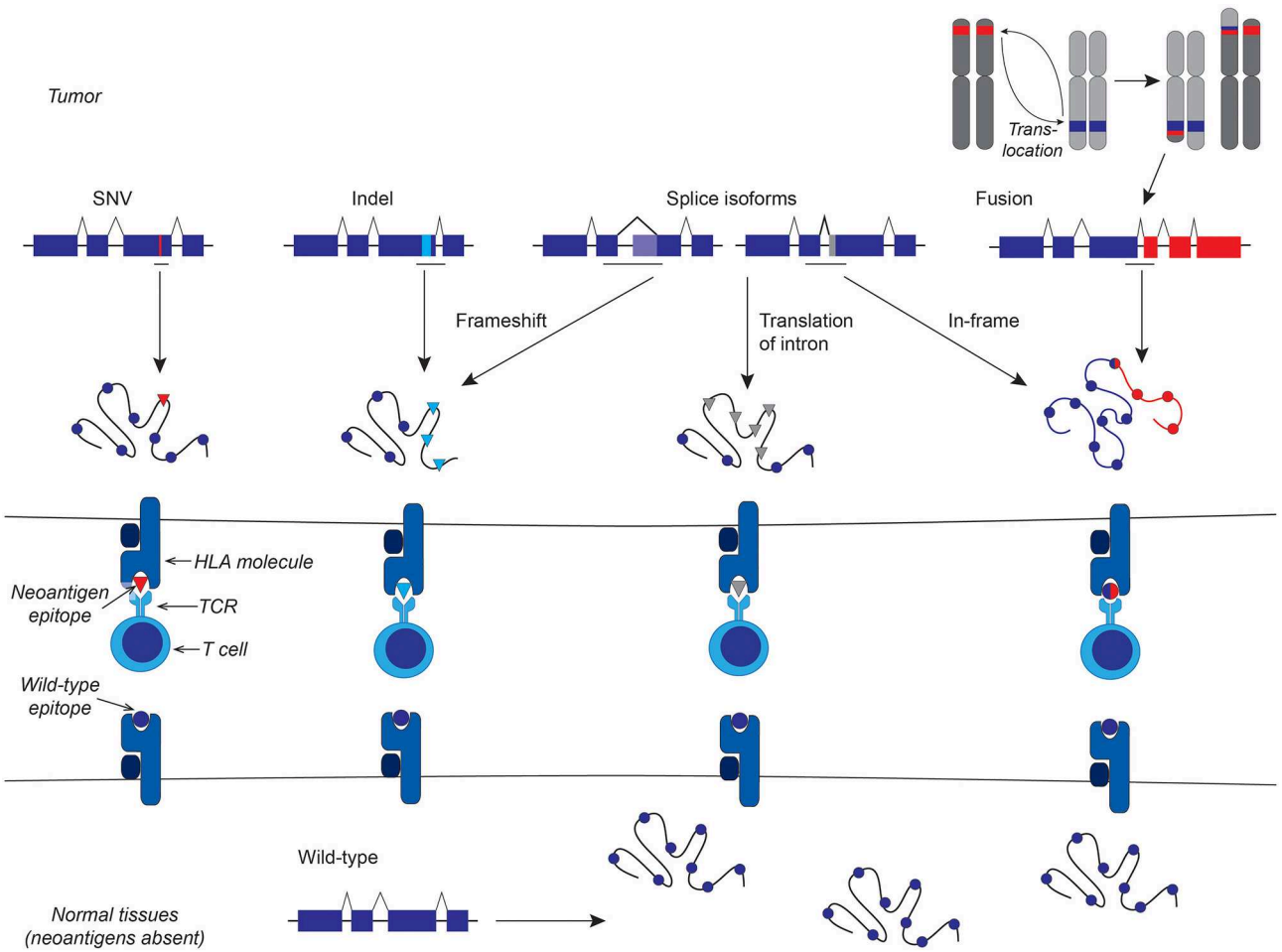

FIGURE 1 | Schematic representation of different classes of non-viral neoantigens. From left to right: Protein-coding single nucleotide variants (SNV) lead to neoantigens that differ from the wild-type antigen by a single amino acid that alters HLA and/or TCR binding. Frameshift insertion-deletion (indels) mutations result in a novel amino acid sequence downstream of the indel. Cancer-specific splice isoforms can lead to frameshifts, if the splice is out of frame, or, like genomic fusions, juxtapose two usually separate amino acid sequences, or produce entirely novel amino acid sequence from introns or other portions of the genome that are not normally translated.

in the founding clone could be curative, and tumor escape through loss of the target protein is unlikely when the neoantigen is from a protein critical for maintaining the malignant phenotype. As neoantigens are presented solely on malignant cells and not on healthy equivalents, the risk of "on-target, off-tumor" toxicity is minimized.

There are three major limitations to therapeutically targeting neoantigens in hematologic malignancies. First, most hematologic malignancies have relatively few protein-coding mutations and/or gene fusions, and thus fewer potential neoantigens than solid tumors, which may carry hundreds or even thousands of mutations in an individual patient (14). Second, therapies directed against any one neoantigen will apply only to the subset of patients who have both the mutation or fusion and restricting HLA allele, making neoantigens a less broadly applicable target than antigens from overexpressed wild-type proteins. However, in contrast to most solid tumors, many hematologic malignancies have recurrent mutations and/or fusions that are common within subgroups of patients and represent shared neoantigens. Finally, though targeting driver-derived neoantigens may prevent escape through loss of the target protein, other mechanism of escape from neoantigen-directed immunotherapy are possible, including downregulation or loss of HLA expression (15–24) or altered proteasomal processing of the epitope (25) by the malignant cell. However, the potential limitations of neoantigens as therapeutic targets are outweighed by their benefits: the high specificity for tumor and absent expression on normal cells; the ability to target intracellular as well as cell surface proteins; and, in some cases, the indispensable role of the aberrant protein in the malignant phenotype (26). Targeting a single high-quality neoantigen can be sufficient for disease control or even cure (4, 6, 7).

NEOANTIGEN DISCOVERY

Innovations in high-throughput genomic and transcriptomic sequencing techniques have greatly facilitated the identification of protein-coding mutations and fusions that produce potential neoantigens. However, there is still no reliable comprehensive *in silico* method for identifying immunogenic neoantigen epitopes from protein-coding mutations, splice variants, or other amino acid sequence-altering abnormalities. One challenge is determining which peptides will be presented on HLA molecules (27). *In silico* HLA-binding prediction algorithms [including but not limited to (28–36)] can predict binding of peptides to HLA molecules with reasonable accuracy and thereby identify candidate neoantigen epitopes. HLA-binding prediction algorithms are quite robust for prevalent HLA class I molecules, and active research by multiple groups has led to a greater understanding of and an improved ability to reliably predict peptide binding to uncommon class I molecules and most class II molecules (37–44). However, HLA-binding predictions do not specify whether the peptides are processed and presented on cell surfaces, although separate predictive tools for antigen processing have been developed (45–53). HLA-binding prediction from the parent amino acid sequence will additionally miss non-canonical epitopes (54), such as post-translationally modified or spliced peptides (55), and will also miss epitopes that are not predicted to bind HLA but actually do (56). An alternative approach is to directly determine the peptidome of malignant cells by immunoprecipitating HLA complexes and then to elute and identify peptides by tandem liquid chromatography–mass spectrometry (57–59). This unbiased approach can identify peptides as they are naturally presented on cells of interest but has significant technical hurdles (60–63). Modifications, such as the use of monoallelic cells (43, 60) should help to overcome some of these technical issues. Since predictive algorithms rely on datasets of peptides that are naturally processed and HLA-binding, improvements to direct identification of HLA ligands will in turn increase the reliability of predictive tools (64).

Determining which epitopes are immunogenic is also a challenge. Presentation of a peptide epitope on an HLA molecule is necessary but not sufficient for T cell recognition. Currently there are no reliable *in silico* tools to assess the immunogenicity of a neoantigen peptide, although this is an active area of research (65–70). There are three starting pools of cells in which one can assess the immunogenicity of a putative neoantigen: patient TIL or marrow-infiltrating lymphocytes (MIL), patient peripheral blood T cells, and healthy donor peripheral blood T cells after primary *in vitro* stimulation. The T cell repertoire of patients may be enriched for neoantigen-specific T cells (71, 72) due to antigen-induced expansion, but immunosuppressive tumor environments can render such T cells dysfunctional (73) or even absent (74). Finally, while neoantigen-specific T cells may exist in the repertoires of patients, unbiased methods to determine the cognate antigens of TCRs from their sequence are still in their infancy (23, 75). Stimulating healthy donor T cells with neoantigen-bearing antigen-presenting cells *in vitro* can be used to isolate reactive T cells without confounding T cell dysfunction (76, 77).

SHARED VS. PERSONAL NEOANTIGENS

Shared or public neoantigens derive from aberrant proteins that are present in all or a sizeable subset of patients with a given disease. In contrast, private or personal neoantigens are those that result from mutations, fusions, or other abnormal amino acid sequences that occur rarely in a disease or are idiosyncratic to an individual's malignancy.

Whole genome and whole exome sequencing of hematologic malignancies (78–85) has revealed the spectrum of fusions and mutations (also referred to as the mutanome) of these diseases, including events that range from rare to highly recurrent. Many of these genetic abnormalities may give rise to neoantigens. Mutanomes provide a rich source of cancer-specific aberrant amino acid sequences that can be interrogated with HLA-binding prediction algorithms to identify candidate neoantigens (70). However, with the exception of one study in the myeloproliferative neoplasms (MPNs) (85), the mutanomes of hematologic malignancies have not yet been thoroughly explored as sources of neoantigens. Another source of both public and personal candidate neoantigens is the HLA peptidome, which is the comprehensive library of peptides eluted from HLA molecules isolated from malignant primary cells and/or cell lines and characterized by mass spectrometry. HLA peptidomes have been defined in acute myeloid leukemia (AML) (57), chronic lymphocytic leukemia (CLL) (59, 86), multiple myeloma (87), and chronic myeloid leukemia (CML) (58). However, mutation-derived candidate neoantigen epitopes have only been identified in more focused HLA peptidome studies [for example, in a subset of AML (74, 88)], reflecting both the heterogeneity of these diseases and the currently limited sensitivity of this approach.

Personal neoantigens can arise from truly patient-specific gene mutations and fusions. In addition, some recurrently affected single genes and gene fusions are highly heterogenous across individuals and would be expected to yield semi-personal rather than shared neoantigens. For example, fusions involving the mixed lineage leukemia (MLL)/ histone-lysine N-methyltransferase 2A (KMT2A) gene in acute lymphoblastic leukemia (ALL) and AML produce diverse amino acid sequences among patients because the fusions may occur at multiple breakpoints in the MLL/KMT2A genes and with multiple (>100) partner genes (89, 90). Recurrently mutated gene in hematologic malignancies that are likely to produce semi-personal rather than shared neoantigens include Wilms tumor 1 (WT1) in AML (91–94) and T cell ALL (95), Notch1 and FBXW7 in T cell ALL (96–98), and TP53 in multiple malignancies (99–101). In these examples, mutations occur at a variety of sites in the gene and involve multiple different nucleotide substitutions, insertions, and/or deletions, such that few, if any, of the resulting amino acid sequences and resulting potential neoantigens would be shared among patients even with the same disease.

At the other end of the spectrum are highly recurrent fusions and mutations, exemplified by the RUNX1-RUNX1T1 fusion (89) and exon 12 mutations in nucleophosmin1 (NPM1) (102, 103) in AML. Virtually all patients with such fusions or mutations will have identical aberrant amino acid sequences. Neoantigens created from these abnormalities are shared among patients who

have the mutations or fusions and are potential therapeutic targets for these individuals as a group.

Whether optimal therapies should target shared neoantigens, personal neoantigens, or both is currently unknown. Some key features of neoantigen quality have been postulated [reviewed in (104)], including: clonality, dissimilarity to self-antigens, similarity to microbial antigens (105), high protein expression, binding to HLA, and low likelihood that genetic abnormality yielding the neoantigen will be lost (for example, driver mutations or genes involved in cell survival where loss would harm cancer fitness) through deletion or transcriptional repression. Neoantigens with high-quality features are likely to be suitable therapeutic targets whether they are personal or shared. One note of caution with personal neoantigens is that unless autologous tumor is available, there may be no way to validate that a given putative neoantigen is in fact presented on primary tumor, and thus no way to confidently predict therapeutic efficacy.

The feasibility of targeting personal neoantigens is currently under investigation. As the accessibility of whole genome and whole exome sequencing increases, defining an individual patient's mutanome is becoming increasingly practical, although the ability to reliably predict personal neoantigens remains imperfect (27). Personalized neoantigen vaccines based on patient mutanomes have shown efficacy in solid tumors (8–10), and as of December 2019, 14 clinical trials of personalized neoantigen vaccines were recruiting in the United States, although only one of these studies includes patients with a hematologic malignancy (NCT03631043, multiple myeloma). In addition, increasingly sophisticated T cell engineering technologies have made the production of personalized neoantigen-specific engineered T cell therapies more practical; currently three trials of such therapies for patients with advanced solid tumors (NCT03412877, NCT04102436, NCT03970382) are enrolling.

Although both personal and shared neoantigens have therapeutic promise, in this review we will focus primarily on shared neoantigens (summarized in **Table 1**), which make up the bulk of the data to date.

NEOANTIGENS IN SPECIFIC HEMATOLOGIC MALIGNANCIES

Acute Myeloid Leukemia

AML is the most common acute leukemia in adults, and mutations in nucleophosmin1 (NPM1) occur in 30–35% of adult patients (102, 103). The majority of NPM1 mutations are insertions of four nucleotides in exon 12, resulting in a frameshift that produces a novel C-terminal 11 amino acid sequence (123). NPM1 mutations are stable across the disease course and considered to be driver events, thus an optimal immunotherapy target. Eighty-five percent of patients with NPM1-mutated ($NPM1^{mut}$) AML share the type A/D mutations that produce an identical abnormal amino acid sequence. Epitopes from the mutated region were independently identified as HLA ligands by two groups that used mass spectrometry to determine the amino acid sequences of peptides eluted off HLA molecules from primary leukemic blasts (74, 88) or AML cell lines (88).

Van der Lee et al. subsequently identified $CD8^+$ T cell clones from healthy donors that were specific for the $NPM1^{mut}$ HLA-A*02:01-restricted epitopes CLAVEEVSL and C*LAVEEVSL (74). These clones specifically recognized HLA-A*02:01^+ peptide-pulsed targets and $NPM1^{mut}$ AML blasts. One C*LAVEEVSL-specific TCR was sequenced and transferred into $CD8^+$ T cells using a viral vector. T cells with transferred $NPM1^{mut}$ TCRs could lyse $NPM1^{mut}$ but not NPM1 wild-type AML *in vitro* and partially controlled leukemia *in vivo* in an $NPM1^{mut}$ OCI-AML3 cell-line-derived xenograft murine model. These results convincingly demonstrate that CLAVEEVSL and C*LAVEEVSL are naturally presented on HLA-A*02:01 on leukemic blasts, are immunogenic, and are thus *bona fide* AML neoantigens. Curiously, although the peptide was immunogenic, the authors were unable to identify naturally occurring epitope-specific T cell responses in HLA-A*02:01^+ patients with $NPM1^{mut}$ AML. While a subsequent publication (123) suggested that $NPM1^{mut}$ -specific responses could be elicited *ex vivo* in patients, these studies were less stringently controlled.

Earlier studies identified candidate $NPM1^{mut}$-derived epitopes predicted to bind HLA-A*02:01 (106–108), against which the authors elicited $CD8^+$ T cells responses in patients and healthy donors after *ex vivo* stimulation. $CD8^+$ T cells specific for two epitopes (AIQDLCLAV and AIQDLCVAV) identified in these publications lysed an $NPM1^{mut}$ AML sample (106), suggesting that these epitopes were naturally processed and presented. However, these peptides were not identified among HLA-A*02:01 ligands in either of two subsequent studies that directly examined peptide epitopes eluted from HLA-A*02:01 on $NPM1^{mut}$ primary blasts (74, 88) or cell lines (88), and identification of AIQDLCL/VAV-specific $CD8^+$ T cells has not been reproduced by other groups.

In around 13% of AML cases (124, 125), a fusion of the retinoic acid receptor (RARA) gene on chromosome 17 and the promyelocytic leukemia (PML) gene on chromosome 15 occurs as a result of the chromosomal translocation, t(15; 17)(q24.1;q21.1), the classic translocation that produces the distinct entity of acute promyelocytic leukemia (APL). The resulting PML-RARA fusion protein not only serves as a driver of the leukemic phenotype but also represents a potential shared neoantigen at the fusion junction. Gambacorti-Passerini et al. investigated the immunogenicity of the PML-RARA fusion region by stimulating peripheral blood mononuclear cells (PBMC) from healthy volunteer donors with a 25mer peptide spanning the fusion (110, 126). $CD4^+$ T cell clones from one donor proliferated specifically in response to exogenous PML-RARA peptide presented on HLA-DR*11 in autologous target cells. One T cell clone could lyse peptide-pulsed autologous target cells and recognize autologous target cells transduced to express the PML-RARA fusion protein. However, in a subsequent study, no PML-RARA-specific $CD4^+$ T cell responses could be elicited from any of four HLA-DR*11^+ individuals in remission after treatment for APL (111). Since neither study evaluated whether PML-RARA-specific T cells could recognize primary APL cells, the PML-RARA/HLA-DR*11 epitope would still currently be

TABLE 1 | Shared or potentially shared neoantigens relevant in hematologic malignancies (fs, frameshift).

Disease	Parent protein	Epitope	HLA restriction	Level of evidence for neoantigen status	Reference(s)
AML	NPM1 fs (type A/D)	C*LAVEESL	A*02:01	Definite	(74, 88)
		CLAVEEVSL			
		AIQDLCLAV		Possible	(106–109)
	NPM1 fs (type C)	AIQDLCVAV		Possible	
	PML-RARA	NSNHVASGAGEAAIETQSSSSEEIV	DR*11	Possible	(110, 111)
ALL	ETV6-RUNX1	RIAECILGM	A*02:01	Conflicting	(112–114)
MPN	CALR fs	KMRMRRMRR	A*03:01	Possible	(85, 109, 115)
		RMRRTRRKM	B*07:02	Possible	
		Multiple	B*08:01	Candidate	(85)
		RMMRTKMRM	C*03:03	Possible	(109)
	JAK2 V617F	VLNYGVCFC	A*02:01	Possible	(116)
	MPL	Multiple	A*03:01	Candidate	(85, 116)
CML	BCR-ABL1	KQSSKALQR	A*03:01	Possible	(117)
	BCR-ABL1 E255K	KVYEGVWKK	A*03:01	Possible	(118)
B cell lymphomas	D393-CD20	(P)LFRRMSSLEVIA	DRB1*04	Possible	(119)
Multiple tumors	KRAS G12D	GADGVGKSA(L)	C*08:02	Definite	(6)
		VVVGADGVGK	A*11:01	Possible	(120)
	KRAS G12V	(V)VVGAVGVGK		Possible	
	BRAF V600E	GDFGLATEKSRWSGS	DQA1*03/ DQB1*03	Possible	(71)
	TP53 R175H	HMTEVVRHC	A*02:01	Possible	(121, 122)
		YKQSQHMTEVVRHCPHHERCSDSDG	Class II	Possible	(121)
	TP53 R248Q	YMCNSSCMGGMNQRPILTIITLEDS	Class I & II	Possible	
	TP53 R248W	SSCMGGMNWR	A*68:01	Possible	
		SSCMGGMNWRPILTII	DPB1*02:01	Possible	
	TP53 R282W	FEVRVCACPGRDWRTEEENLRKKGE	Classs II	Possible	

For levels of evidence:
"Definite" indicates that the epitope is immunogenic and that epitope-specific T cells clearly and consistently recognize primary malignant cells in a mutation/fusion- and HLA-specific manner.
"Possible" indicates epitopes that have demonstrated immunogenicity but either lack direct evidence of specific recognition of primary malignant cells (i.e., cell lines only) or data is inconsistent.
"Candidate" indicates that the peptide epitope has been demonstrated to bind the restricting HLA in vitro.
"Controversial" indicates conflicting data between groups.

considered a possible, rather than definite, AML neoantigen pending confirmation that the epitope is naturally presented on APL cells.

Acute Lymphoblastic Leukemia

ALL is the most common childhood cancer. Like other hematologic and pediatric malignancies, there are few non-synonymous mutations (14, 127) and thus few potential neoantigens. However, in recent studies, Zamora et al. found surprisingly abundant neoantigen-specific $CD8^+$ T cell responses in MIL from pediatric patients with ALL (112). To identify putative patient-specific neoantigens, cancer-specific mutations were identified from genomic sequencing of diagnostic biopsies and matched germline tissues from six patients. HLA typing was extrapolated from sample mRNA sequencing data, and the amino acid sequences of protein-coding mutations were interrogated using HLA-binding prediction algorithms. Mutation- or fusion-derived 15mer synthetic peptides were used to evaluate patient T cell specificity *ex vivo*. Functional $CD8^+$ T cell responses against at least one neoantigen were detected in all patients and encompassed 31 of 36 putative neoantigens mostly originating from patient-specific single gene mutations.

The Zamora study also identified T cells responsive to several epitopes from the recurrent ETV6-RUNX1 fusion in five patients. The ETV6-RUNX1 fusion results from the t(12;21)(p13.2;q22.1) chromosomal translocation and is the most common genetic event in childhood B-lineage ALL, occurring in 15–20% of patients (128–131). ETV6-RUNX1 epitopes eliciting T cell responses in this study were predicted to bind to HLA-A*02:01, HLA-A*11:01, and HLA-B*15:01, and ETV6-RUNX1-specific T cells were identified by positive staining with HLA-A*02:01 or HLA-A*11:01 peptide/HLA tetramers. In earlier

studies, the same HLA-A*02:01 epitope (RIAECILGM) was identified as binding stably to HLA-A2 in *in vitro* competitive-binding assays by a group that also isolated $CD8^+$ T cell lines specific for the epitope (113). The two RIAECILGM-specific lines that were isolated from healthy donors lysed fusion-expressing cell lines, and one T cell line from a patient with ETV6-RUNX1$^+$ ALL lysed autologous leukemic blasts at low levels. However, a subsequent study disputed whether the ETV6-RUNX1 epitope is in fact naturally processed and presented, as it showed that the native RIAECILGM peptide had virtually no binding to HLA-A*02:01 *in vitro*, was not processed by cells transduced to express the ETV6-RUNX1 epitope, and was not cleaved at the relevant position by human proteasomes *in vitro* (114). Given the conflicting data, it remains unclear whether the RIAECILGM epitope is truly an ALL neoantigen.

Myeloproliferative Neoplasms

Philadelphia (Ph) chromosome-negative myeloproliferative neoplasms (MPNs) comprise a group of disorders, including essential thrombocytosis (ET), polycythemia vera (PV), and primary myelofibrosis (PMF). MPNs arise from an abnormal hematopoietic progenitor cell, in most cases consequent to the acquisition of one of three driver mutations in JAK2 (Janus kinase 2), CALR (calreticulin), or MPL (c-mpl proto-oncogene; thrombopoietin receptor), along with a variety of passenger mutations (132) that can all produce neoantigens. Recently, Schischlik et al. comprehensively evaluated potential neoantigens in 113 patients with MPNs (85). Using whole-transcriptome sequencing to define the MPN mutanome, they identified 13 fusions, 221 non-synonymous single nucleotide variants, 31 insertion or deletion mutations, and 20 frameshift-producing splicing abnormalities. HLA-binding predictions for the 12 most prevalent HLA-A, -B, and -C alleles in their patient cohort yielded 541 patient-specific peptides predicted to bind to at least one of the HLA alleles. Subsequent *in vitro* HLA binding studies of 35 peptides derived from aberrantly spliced proteins associated with SF3B1 mutations and from mutated CALR ($CALR^{mut}$) and MPL validated binding of 23 peptides to HLA-A*03:01, -A*11:01, -B*07:02, and -B*08:01.

Although Schischlik et al. did not evaluate processing or immunogenicity of their putative neoantigens, others have identified T cell responses to $CALR^{mut}$ and JAK2 V617F. Cimen Bozkus et al. used *in vitro* stimulation to elicit T cell responses to $CALR^{mut}$ peptides that were primarily $CD4^+$ T cells in patients with MPNs and both $CD4^+$ and $CD8^+$ in healthy donors. Inhibition of the PD-1 and CTLA-4 immune checkpoint molecules *in vitro,* and PD-1 *in vivo* (in a patient treated with pembrolizumab), enhanced T cell responses. An immunogenic HLA-C*03:03-restricted 10mer epitope was identified, and T cells specific for this epitope produced cytokine in response to antigen-presenting cells pulsed with a 15mer peptide, indicating that the epitope was processed from the longer peptide (109). While this finding is encouraging, data conclusively demonstrating that the $CALR^{mut}$ epitope is processed from the full-length protein and presented on HLA-C*03:03 on primary MPN cells is currently lacking. Another group described cytokine production, primarily by $CD4^+$ T cells, in response to *ex vivo* stimulation of peripheral blood mononuclear cells from patients with MPNs with long (31mer) $CALR^{mut}$ peptides (133). $CD8^+$ T cells specific for $CALR^{mut}$ peptides presented on HLA-A*03:01 and -B*07:02 were identified by another group, but the low avidity of the T cells prevented them from assessing whether the epitopes were naturally processed and presented on $CALR^{mut}$ cells (115). Additionally, a 9mer peptide spanning the JAK2 V617F mutation (VLNYGVCFC) was identified as a ligand of HLA-A*02:01 by HLA-binding prediction; epitope-specific $CD8^+$ T cells lysed target cells either pulsed with the mutant peptide or naturally expressing JAK2 V617F, but also recognized targets pulsed with wild-type JAK2 peptide with lower efficiency (116). While this is a promising possible neoantigen with broad applicability for patients with MPNs, especially PV, further study is needed to definitively show that VLNYGVCFC is presented on primary malignant cells.

Philadelphia Chromosome-Positive Malignancies

The BCR-ABL1 fusion derives from translocation t(9; 22)(q34;q11), also called the Ph chromosome, which is highly recurrent in chronic myeloid leukemia (CML) and Ph-positive ALL (Ph^+-ALL). Most patients have one of two fusions resulting from different breakpoints, namely ^{p210}BCR-ABL1 and ^{p190}BCR-ABL1. ^{p210}BCR-ABL1 is found in both CML and Ph^+-ALL, while ^{p190}BCR-ABL1 is primarily associated with Ph^+-ALL (134). As an oncogenic driver, the BCR-ABL1 fusion is essential to the malignant phenotype and an ideal therapeutic target. Small molecule tyrosine kinase inhibitors (TKIs) are now key components of therapy for Ph^+ malignancies, but resistance does occur. Because the fusion is highly recurrent and disease-specific, it is a potential source for shared neoantigens. BCR-ABL1 was first described as a neoantigen in 1992 (135), and additional BCR-ABL1 epitopes were subsequently investigated by multiple groups [reviewed in (136)]. However, evidence for the natural CML presentation of BCR-ABL1 fusion peptides is conflicting: one group eluted an immunogenic fusion peptide from HLA-A*03:01 in primary CML (117), but a recent comprehensive evaluation of the HLA-ligandome in CML found no BCR-ABL1 epitopes presented on class I or class II molecules (58). Because the biology and specific fusions differ in the two diseases, the CML and Ph^+-ALL peptidomes may differ. Interestingly, adoptive transfer of *ex vivo*-expanded ^{p190}BCR-ABL1-specific $CD8^+$ T cells showed encouraging anti-leukemic activity in three patients with Ph^+-ALL (137). Specific BCR-ABL1 mutations that confer TKI resistance might also serve as neoantigens; one group identified donor-derived $CD8^+$ T cell responses to an HLA-A*03:01-restricted epitope from BCR-ABL1 E255K in a patient with the mutation who had achieved remission after HCT (118). While the BCR-ABL1 E255K-specific T cell clones could recognize minigene-transduced target cells, recognition of primary CML was not tested and thus it remains unclear whether the epitope represents a *bona fide* CML neoantigen.

Lymphomas and Chronic Lymphocytic Leukemia

In B cell malignancies, such as lymphomas and myelomas, neoplastic B cell-produced clonal immunoglobulin (Ig) was first described as a tumor-specific antigen in 1972 (138). Ig idiotypes have been extensively investigated as neoantigens with varying degrees of success, including in clinical trials (139). More recently, Khodadoust et al. recovered peptides representing somatic mutations in Ig heavy and light chain genes from the peptidomes of both class I and class II molecules in 17 primary mantle cell lymphomas (MCL) and two MCL cell lines, and detected circulating functional $CD4^+$ T cells specific for one Ig neoantigen that could kill autologous lymphoma (140). Subsequent studies by this group identified primarily class II-restricted Ig-derived neoantigens in other B cell malignancies, including follicular lymphoma, diffuse large B cell lymphoma, and chronic lymphocytic leukemia (CLL) (141). A cytoplasmic variant of CD20 (D393-CD20), produced by alternative splicing of the CD20 transcript, is detectable in malignant primary B cells and B cell lines, but not normal resting B cells (142). $CD4^+$ T cell responses to an epitope of D393-CD20 could be elicited from both healthy donors and patients with B cell lymphomas after *in vitro* peptide stimulation and blocked with anti-HLA-DR monoclonal antibody, but the exact HLA restriction could not be determined (119). *MYD88* is recurrently mutated in a variety of B cell malignancies and has been proposed as a potential neoantigen (143). Separately, in a small cohort of CLL patients evaluated after HCT, $CD8^+$ responses to neoantigens created from patient-specific non-Ig somatic mutations were identified; one well-studied patient-derived T cell clone could lyse autologous primary CLL cells, indicating that the epitope the clone recognized was a true personal neoantigen (13).

Neoantigens With General Applicability in Hematologic Malignancies

While some genetic abnormalities are specific to or even defining of particular cancer types, others, especially mutations in oncogenes or tumor suppressor genes, can be found in numerous cancers with a wide variety of cellular origins, including hematopoietic tissues. For example, somatic mutations affecting members of the Ras-MAPK pathway are among the most common in human cancers and are found across diverse cancer types (144–147). Similarly, TP53 is the most commonly mutated gene in human cancer, with TP53 mutations estimated to occur in ∼25% of all cancers (99). Neoantigens derived from these mutations may thus be shared not just among patients with a single disease but across patients with many different cancers, including hematologic cancers (**Table 2**).

Mutations in KRAS or NRAS are found in ∼5–26% of hematologic malignancies (146, 148) (**Table 2**). The most recurrent oncogenic mutations that occur in the RAS genes (NRAS, KRAS, HRAS) across cancers occur at codons 12, 13, and 61. As such, neoantigens derived from these recurrent mutations in RAS genes are attractive therapeutic targets with applicability in multiple diseases, including blood cancers. Moreover, the amino acid sequences of RAS family members are highly similar, such that identical epitopes may be derived from different proteins. Although there are no publications specifically investigating RAS-derived neoantigens in hematologic malignancies, findings from studies in solid tumors have potential applicability. For example, Tran et al. studied tumor-infiltrating lymphocytes (TIL) from a patient with metastatic KRAS G12D-mutated colorectal cancer and identified $CD8^+$ T cell clones specific for a KRAS G12D epitope presented on HLA-C*08:02 (6). KRAS G12D specific T cells expanded in the patient's peripheral blood after re-infusion of TIL, were persistently detectable ∼9 months after TIL infusion, and mediated at least a transient regression of metastatic lung lesions. Subsequently, Cafri et al. performed *in vitro* stimulation of memory T cells isolated from two patients with KRAS-mutated solid tumors (one with endometrial cancer, one with rectal cancer) and identified $CD8^+$ T cells specific for an HLA-A*11:01-restricted epitope from KRAS G12V and $CD4^+$ T cells specific for an HLA-DRB1*08:01 restricted epitope from KRAS G12D (156). Earlier studies also identified murine TCRs with specificity for HLA-A*11:01-restricted epitopes from KRAS G12D and G12V in HLA-A*$11:01^+$ transgenic mice immunized with KRAS peptides (120). Retroviral transfer of the KRAS-specific TCRs into human T cells conferred KRAS neoantigen-specific anti-tumor activity *in vitro* and *in vivo*. These findings have been translated into clinical trials of transgenic TCR T cell immunotherapy for HLA-A*$11:01^+$ patients with certain KRAS G12D- or G12V-mutated solid tumors (NCT03190941 and NCT03745326). Although this clinical trial is directed toward patients with solid tumors, such therapies also have applicability to those with hematologic malignancies; for example, alterations at codon G12 of NRAS occur in a subset of patients with AML and produce identical amino acid sequences to the equivalent KRAS mutations, and so should yield the same epitope that could be targeted with KRAS G12D or G12V-directed T cells.

Mutations in BRAF, another component of the Ras-MAPK pathway, are present in about 8% of all human cancers (150). While the majority of BRAF-mutated malignancies are solid tumors, BRAF mutations do occur in a subset of hematologic malignancies. The BRAF V600E mutation is highly prevalent in hairy cell leukemia (151–153) and systemic histiocytoses (Erdheim-Chester disease and Langerhans cell histiocytosis) (154) and have also been identified in CLL (148) (**Table 2**). BRAF-derived neoantigens, particularly those originating from the V600E mutation, thus have applicability in a subset of hematologic malignancies. By examining peripheral blood lymphocytes from a patient with BRAF $V600E^+$ melanoma who had a clinical response after TIL therapy, Veatch et al. identified a $CD4^+$ T cell clone specific for an HLA-DQB1*03-restricted epitope of BRAF V600E (71). Lentiviral transfer of the BRAF V600E-specific TCR to donor $CD4^+$ conferred recognition of BRAF V600E-expressing target cells. An earlier study also detected $CD4^+$ T cell responses to HLA class II-restricted epitopes from BRAF V600E in patients with BRAF V600E melanoma, although not in the context of clinical response after immunotherapy (157).

TP53 mutations occur in malignancies of all origins (99), including all types of hematologic malignancies (100, 101, 158) (**Table 2**). While TP53 mutations can be quite heterogeneous,

TABLE 2 | Recurrently mutated genes in cancers, including hematologic malignancies, for which possible or definite public neoantigens have been identified.

Gene/gene family	Overall prevalence of any mutation in the gene/gene family in human cancers	Prevalence of any mutation in the gene/gene family in hematologic malignancies	Mutation hotspots (all cancers including hematologic)	Mutations yielding possible/definite neoantigens*	References
KRAS/ NRAS/HRAS	∼25% (all RAS genes)	∼26% multiple myeloma ∼16% AML ∼14% ALL ∼10% CLL ∼5% MDS (∼30% CMML)	G12, G13, Q61	G12D, G12V	(146, 148, 149).
BRAF	∼8%	∼100% hairy cell leukemia ∼40–60% systemic histiocytoses ∼5% CLL	V600	V600E	(148, 150–154)
TP53	∼25%	14% AL 12% AML 7–10% CLL 6% MDS 6–24% B cell lymphoma 7–40% non-B cell lymphoma 6% myeloma and other plasma cell dyscrasias	R175, R245, R248, R273, R282	R175H, R248Q, R248W, R282W	(99–101, 155)

*See **Table 1** for specific details about neoantigens.*

there are mutation hotspots at R175, R245, R248, R273, and R282 that are shared across multiple kinds of cancers, including hematologic cancers (99, 101). Neoantigens created from TP53 mutations thus have broad potential applicability in blood cancers as well as solid tumors. Malekzadeh et al. isolated T cells specific for HLA class I- and class II-restricted epitopes from five different recurrent TP53 mutations from TIL generated from patients with a variety of epithelial tumors (colorectal, ovarian, and pancreatic) (121). Both this publication and a subsequent report from the same group (122) identified an HLA-A*02:01-restricted epitope from TP53 R175H that appears to be naturally presented on a number of tumor cell lines. $CD4^+$ and $CD8^+$ responses to epitopes from patient-specific TP53 mutations have also been identified (122, 159, 160). Because the codon distribution of TP53 mutations is not specific to the tissue origin of a cancer, therapy targeting the TP53 R175H epitope, for example, should be equally applicable in a TP53 $R175H^+$ hematologic cancer as in a TP53 $R175H^+$ solid tumor, assuming the epitope is processed and presented appropriately. Similarly, specific mutations that are identical in many different malignancies, like the ones described in this section, are sources for neoantigens that are shared across cancers.

THERAPEUTIC APPLICATIONS OF NEOANTIGENS

While neoantigens are attractive targets for therapy because of their high specificity for malignant cells, there are challenges in translating neoantigen-directed immunotherapies to the clinic and the best approach to neoantigen-directed therapy is currently unknown. One strategy is to adoptively transfer neoantigen-specific T cells. T cells can be isolated from patient peripheral blood, TIL, or MIL (in hematologic malignancies), then expanded *ex vivo* non-specifically or against a defined antigen and re-infused (4, 6, 7, 161). Alternatively, T cells can be engineered to express a transgenic neoantigen-specific TCR (TCR-T), allowing infusion of a rapidly generated product with defined specificity and composition. Preclinical studies have shown that transfer of neoantigen-specific TCR-Ts is feasible (71, 74, 122), and two clinical trials of autologous TCR-T targeting HLA-A*11:01-restricted epitopes derived from point mutations in KRAS are enrolling (NCT03190941 and NCT03745326). Although TCR-T targeting epitopes from wild-type WT1 have shown safety (162–164) and efficacy (165), no clinical trials of neoantigen-specific TCR-T immunotherapy for hematologic malignancies have opened to date. TCR constructs can be modified to include other features to improve TCR-T safety and function: a CD8 co-stimulatory receptor enables $CD4^+$ T cells to function with a class I-restricted TCR and provide targeted help to neoantigen-specific $CD8^+$ T cells (166–168), a safety switch (167, 169) enables rapid removal of transgenic TCR-T cells in the event of toxicity, and other elements have been advanced [reviewed in (170)]. Lastly, vaccines do not require adoptive cell transfer, have shown clinical efficacy in solid tumors (8–10), and are particularly attractive for targeting highly immunogenic but less prevalent neoantigens.

Many factors influence which immunotherapy strategy is optimal for a given antigen. For neoantigens, the relatively low prevalence of each neoantigen among individuals with a given hematologic malignancy is a significant consideration, as immunotherapy for one neoantigen will apply only to a subset of patients. For example, the $NPM1^{mut}$ epitope

described above (74) is only presented by the ∼15% of AML patients with $NPM1^{mut}$ (30–35%) and HLA-A*02:01 (∼50% in the U.S.A.) and this represents one of the most broadly applicable recurrent neoantigens in hematologic malignancies. Producing neoantigen-directed TCR-T therapies using currently standard viral transfer methods is probably not cost-effective for less common neoantigens given their narrow applicability, but vaccines could be. Moreover, the growing use of non-viral techniques for TCR gene transfer, such as transposon-based technologies (159, 171, 172), nanoparticles (173), and RNA electroporation (174) should facilitate the development of TCR-T immunotherapy for all neoantigens, as illustrated by a recently opened clinical trial of gene-edited TCR-T immunotherapy for personal neoantigens (NCT03970382). The use of "universal donor cells" that have been engineered to be HLA-negative and express natural killer (NK) cell inhibitory molecules (175), in combination with silencing or editing the endogenous TCR (163), could also facilitate neoantigen-directed TCR-T immunotherapy.

Another consideration is the natural immunologic landscape of a particular malignancy. *Ex vivo* expansion and vaccination rely largely on the presence of existing anti-tumor responses that can be boosted *in vivo* or *ex vivo* and would be challenging in an immunosuppressive environment. Because hematologic malignancies have multiple mechanisms for blocking effective naturally occurring anti-leukemic immune responses (11, 176–183), TCR-T immunotherapy may be preferable for these diseases. For example, transgenic TCRs can used to modify selected virus-specific memory T cells for therapeutic transfer (165). Immune checkpoint blockade has been used alone in MPNs (109); combining them with neoantigen-specific immunotherapies could potentiate their effect in these and other hematological malignancies.

DISCUSSION

Much progress has been made in the field of neoantigens generally and in hematologic malignancies specifically. A number of promising *bona fide* and potential shared neoantigens have been identified for hematologic malignancies, most of which are derived from well-established mutations and fusions. However, growing access to comprehensive sequencing technologies has greatly enhanced the ability to define disease- and patient-specific mutanomes, which are valuable sources of potential neoantigens. Combined with improvements in T cell antigen discovery approaches, sequencing advances will facilitate the discovery of additional shared and personal neoantigens derived from known as well as new genetic abnormalities, expanding the repertoire of potential targets and moving the field forward. While there are a number of challenges in translating neoantigen-directed immunotherapies to the clinic, the rapid evolution of neoantigen discovery methods and the immunotherapy field is making barriers to clinical translation surmountable. Experience gained from T cell immunotherapy and vaccine studies in solid tumors and from cell therapy engineering for non-neoantigen targets will provide a critical foundation for building potent neoantigen-directed immunotherapies that are viable treatment strategies for hematologic malignancies.

AUTHOR CONTRIBUTIONS

MAB and MB reviewed the literature and wrote and edited the manuscript.

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REVIEW
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T-Cell Immunotherapies Targeting Histocompatibility and Tumor Antigens in Hematological Malignancies

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Over the last decades, T-cell immunotherapy has revealed itself as a powerful, and often curative, strategy to treat blood cancers. In hematopoietic cell transplantation, most of the so-called graft-vs.-leukemia (GVL) effect hinges on the recognition of histocompatibility antigens that reflect immunologically relevant genetic variants between donors and recipients. Whether other variants acquired during the neoplastic transformation, or the aberrant expression of gene products can yield antigenic targets of similar relevance as the minor histocompatibility antigens is actively being pursued. Modern genomics and proteomics have enabled the high throughput identification of candidate antigens for immunotherapy in both autologous and allogeneic settings. As such, these major histocompatibility complex-associated tumor-specific (TSA) and tumor-associated antigens (TAA) can allow for the targeting of multiple blood neoplasms, which is a limitation for other immunotherapeutic approaches, such as chimeric antigen receptor (CAR)-modified T cells. We review the current strategies taken to translate these discoveries into T-cell therapies and propose how these could be introduced in clinical practice. Specifically, we discuss the criteria that are used to select the antigens with the greatest therapeutic value and we review the various T-cell manufacturing approaches in place to either expand antigen-specific T cells from the native repertoire or genetically engineer T cells with minor histocompatibility antigen or TSA/TAA-specific recombinant T-cell receptors. Finally, we elaborate on the current and future incorporation of these therapeutic T-cell products into the treatment of hematological malignancies.

Keywords: histocompatibility antigens, tumor-specific antigens (TSA), tumor-associated antigens (TAA), transgenic T-cell receptors, T-cell immunotherapy, viral antigens, allogeneic stem cell transplant, chimeric antigen receptor (CAR)

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INTRODUCTION

Allogeneic hematopoietic cell transplantation (AHCT) remains to this day the most widely used form of cancer cellular immunotherapy. Several studies in both humans and animals have conclusively shown that the recognition of alloantigens by T cells is central to the so-called "graft-vs.-tumor" (GVT) that occurs following AHCT (1–3). However, the recognition by donor

T cells of major and minor histocompatibility antigens (MiHA), encoded by germline polymorphisms and expressed on malignant and normal host hematological cells as well as on non-hematological cells, can also result in graft-vs.-host disease (GVHD) (4). Despite several decades of research, the potentially lethal GVHD reactions are still the major limitation to the use of alloreactivity to treat blood cancers with AHCT. Recent antigen identification and characterization methods, coupled with refined cell manipulations and cell transfer procedures, may allow for an effective separation of the GVT and GVHD effects when targeting alloantigens. Moreover, other antigens are inspiring immunotherapeutic strategies that can be implemented in AHCT and non-transplant settings (5). The tumor-specific antigens (TSA) refer to major histocompatibility complex (MHC) class I or II-associated peptides that are found solely at the surface of tumor cells. Often resulting from acquired genetic variants, these antigens can stimulate vigorous T-cell responses and will be extensively described below. T-cell immunotherapies targeting unmutated MHC-associated antigens, including viral antigens and tumor-associated antigens (TAA) will also be described in the context of blood cancers. This review will focus on the current status of immunotherapeutic approaches, particularly those exploiting genetic variants, native and acquired, for the treatment of hematological malignancies. These antigens are almost exclusively relevant to the context of immune therapies using conventional T cells, $CD8^+$ and $CD4^+$, that recognize their MHC-bound peptide antigen through a T-cell receptor (TCR) composed of an alpha and beta chain (**Figure 1**). T-cell therapies targeting non-polymorphic antigens and the use of other immune cell types will also be briefly discussed and put in context of the current status of cellular immunotherapies for blood neoplasms. The implementation of T-cell therapies targeting relevant antigens for hematological cancers hinges on a detailed knowledge of the targets, T-cell biology, gene engineering, *ex vivo* cell processing methods and clinical expertise. As such, these therapies represent a formidable challenge but also an opportunity to make paradigmatic advances in blood cancer treatment and oncology in general.

TARGET ANTIGENS IN HEMATOLOGICAL CANCERS

Histocompatibility Antigens, Majors, and Minors

AHCT's curative potential relies substantially on the GVT effect, which is largely based on the recognition of histocompatibility antigens by allogeneic T cells. These antigens result from the translation of germline-encoded genetic variants (6–10). However, standard AHCT is a personalized but markedly unspecific form of immunotherapy. The broad repertoire of allogeneic T cells transferred with the graft react against a multitude of host derived antigens. These can be expressed on several cell and tissue types, inducing GVHD in most recipients despite prophylactic immunosuppression (11, 12). Thus, the curative potential of AHCT relies on the transfer of histo-incompatible T cells recognizing germline genetic variants on neoplastic cells (13–17). Histocompatibility antigens are prime targets for T cells because they stimulate a high avidity T-cell repertoire. Histocompatibility antigens are not expressed in donor thymus, therefore T cells recognizing histocompatibility antigens with high functional avidity do not undergo negative selection prior their adoptive transfer in patients (18, 19). Moreover, the high frequency of GVHD occurrence in recipient of multiparous female donors hints at the possibility of sensitization to host recipient antigens and the mobilization of a memory T-cell repertoire against these antigens (20). Thus, AHCT patients receive a treatment which is targeted to a mostly unknown set of antigens by an equally elusive T-cell repertoire leading to frequent toxic "on-target/off-tumor" immune responses. The discovery and characterization of relevant transplantation antigens nonetheless hold great promise for the design of immunotherapies that could enhance the GVT effect and limit the occurrence of GVHD. The development of such immunotherapies depends on the identification of antigens that are specifically, or at least preferentially, expressed on hematopoietic and/or malignant cells (6, 21). As such, Human leukocyte antigen (HLA) (the major histocompatibility antigens) and MiHA mismatches can be harnessed to treat hematological cancer patients.

The frequency of T cells capable to target mismatched HLA molecules is very high (1–10%) (22–24). Given the likelihood of severe GVHD occurrence when AHCT is performed across HLA barriers, refinements in HLA typing in the last years have improved outcomes due to better matching (25, 26). To this day, HLA compatibility remains a key variable in AHCT and most centers consider that a related or unrelated HLA identical donor is the best donor. However, recent advances in cell handling and GVHD prophylaxis now enable the use of partially HLA mismatched cord blood and related haplo-identical donors, with results that are comparable to those obtained with HLA matched donors (27, 28). In both cases, the risk of GVHD (especially chronic GVHD) is surprising low. Although the reasons for this are incompletely understood, several factors, such as the intensity of the immunosuppression in haplo-identical AHCT, or the intrinsic features of the graft in terms of cell composition and functionality in cord blood transplants, may contribute to this observation (29, 30). Moreover, in certain circumstances, the risk of relapse appears to be lower following these mismatched transplants, arguing in favor of enhanced GVT in these settings (31–33). Based on the presumption that anti-HLA T-cell reactivity is an effective anti-neoplastic mechanism, the infusion of intentionally mismatched peripheral blood mononuclear cells following chemotherapy is being investigated as a form of immunological consolidation after chemotherapy (34). Such "microtransplantations" resulted in improved leukemia outcomes relative to the usually reported survival and leukemia-free survival rates, despite the absence of prolonged and significant engraftment (35, 36). These results need nonetheless to be confirmed and the underlying mechanisms better defined. Several questions remain about the relative contribution of $CD4^+$ and $CD8^+$ HLA-specific T cells and other cell types, such as natural killer (NK) cells in the recognition of HLA-mismatched cellular targets

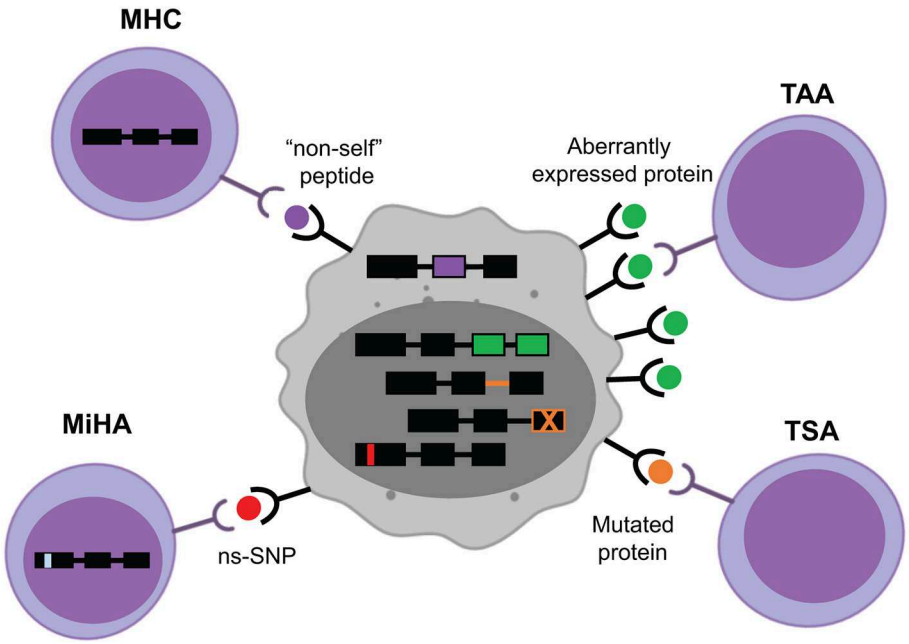

FIGURE 1 | Target MHC-associated antigens in hematological cancers. Major histocompatibility complex (MHC)-associated antigens may originate from viral components, such as the episomal translation of Epstein-Barr Virus proteins (purple). The majority of known minor histocompatibility antigens (MiHA) are generated by non-synonymous single nucleotide polymorphisms (ns-SNP) between the donor and the recipient of the T-cell therapy (red). Tumor-specific antigens (TSA) arise from intronic or exonic mutations unique to the tumor cells (orange). Tumor-associated antigens (TAA) come from aberrantly expressed proteins in cancer cells (green).

[reviewed in Paul and Lal (37)]. The infusion of HLA mismatched NK cells has led to promising clinical results, confirming a direct anti-neoplastic effect (38, 39). Hence, the respective impacts of T-cell and NK-cell reactivity in HLA mismatched transplants and other cell therapy approaches are still unknown but may account for the effects on GVT and GVHD observed in haplo-identical and cord blood transplants. To this date, no clinical studies using *ex vivo* expanded anti-HLA reactive T cells have been reported. Although this may be fraught with the risk of inducing severe GVHD, the design of anti-HLA T-cell therapy targeting the class II antigens which have a more restricted tissue expression (with high expression in blood cancer subtypes) may be considered (40, 41).

In the context of HLA-matched AHCT, alloreactive donor T cells ($CD4^+$ and $CD8^+$) recognize MHC-bound polymorphic peptides derived from the host proteome and known as the MiHA. Both MHC class I and class II molecules have been shown to present MiHA (2, 6, 42–47). Most of the molecularly characterized MiHA are encoded by autosomal genes that differ between patient and donor secondary to germline encoded non-synonymous single nucleotide polymorphisms (ns-SNP). However, the true contribution of ns-SNP to MiHA disparities is unknown. Several non-SNP events, such as alternative proteasome degradation, non-presentation of allelic variants, Y-chromosome derived peptides, polymorphic proteins created by frameshift insertions or deletions [reviewed in Griffioen et al. (48)] can also generate MiHA. However, these are more difficult to characterize using currently available methods (45, 49). Recent evidence suggest that the genetic origin of the MiHA presented by MHC class I is not random, with specific exomic regions coding for proteins being overrepresented among the repertoire of MHC-peptides directly assessed by proteomic methods (45, 50). This indicates that relying on ns-SNP detection to predict MiHA's sequences is fraught with limitations as only 0.5% of ns-SNP generate MHC-associated peptides (6, 51). More comprehensive proteogenomic analyses, including the direct identification of MHC-associated peptides by mass spectrometry appears to be required to permit the robust, and high-throughput, identification of candidate MiHA that derive from ns-SNP (6, 51). One shortcoming of current methods to define MHC-associated peptides as candidate antigens, is that our current proteomics and bioinformatics tools are better at identifying MHC class I than class II-associated peptides (52, 53). In addition to the confirmation of presentation by the MHC, candidate MiHA for immunotherapy should fulfill several other criteria (**Figure 2**). Perhaps the most important is tissue restriction. In the context of AHCT, the expression of the source protein of a given MiHA should be restricted to the hematopoietic system and the malignant cells. Determining tissue distribution can be achieved through several methods including bioinformatics mining of tissue gene expression atlases to standard biochemical and histological methods (6). Moreover, a practical consideration is that the minor allele frequency (MAF) of a MiHA sequence should be well-balanced in the population to enhance the odds that there will be a mismatch between the donor and recipient. Pre-clinical studies in mice demonstrated the curative potential

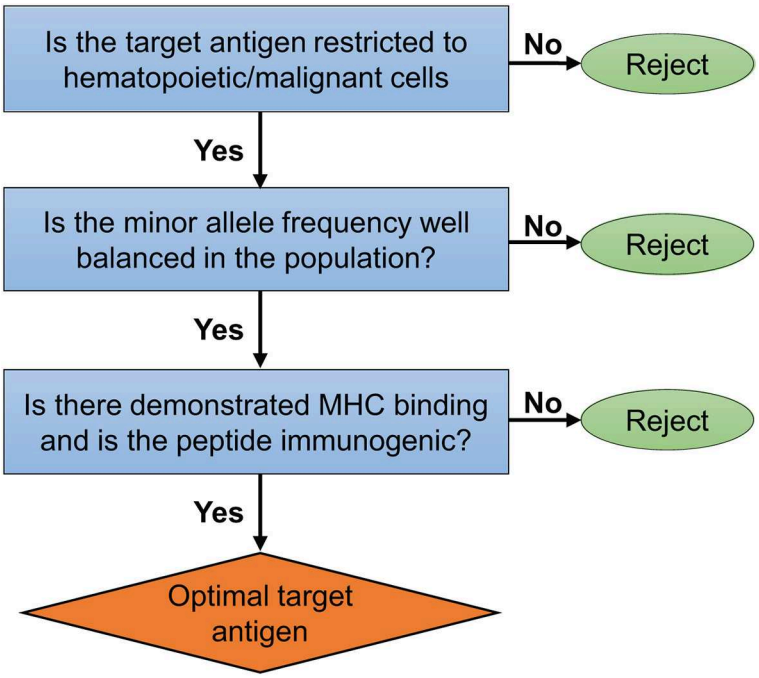

FIGURE 2 | Ideal MiHA target selection. Important criteria and proposed algorithm to select optimal target MiHA for immunotherapy of blood cancers.

(without causing GVHD) of injected T cells primed against a single MiHA (54–56) offering solid proof of principle for the development of MiHA-based immunotherapeutics in humans.

Tumor-Specific Antigens (TSA)

The genetic mutations that characterize the neoplastic process can result in acquired ns-SNP with altered reading frames and the translation of proteins with different amino acid sequences (57). Once degraded and presented by MHC molecules at the cell surface, these altered sequences can be recognized by the host T cells. Since the mutations giving rise to these so-called neoepitopes are present only in cancer cells, the resulting antigens are deemed specific for the tumor. TSA are therefore thought to be most prevalent in highly mutated cancers, such as melanoma and lung cancer. The correlation (albeit very imperfect) between mutation load and responses rates to immune checkpoint (CTLA-4 and PD-1) blockade reinforces the notion that the mutanome is immunologically relevant (6, 58–60). The discovery of TSA has provided new hopes for the field of cancer vaccines with several trials launched in the last decade (61–66). It has also provided a rationale to explain the success of tumor infiltrating lymphocytes (TIL) infusions in certain cancers (67). The identification of putative patient-specific tumor antigens generated by somatic mutation is unfortunately insufficient as most mutations identified in tumor-expressed genes do not generate neoepitopes capable of stimulating T-cell responses. It has been estimated that only 10% of the non-synonymous mutations in tumor cells can generate mutant peptides with high MHC affinity (68), while only 1% of peptides with high MHC affinity can be recognized by T cells in patients (69). Moreover, a large fraction of these mutations are not shared between patients and often not by all cells comprised in the tumor or metastases (70, 71). Such heterogeneity forces the development of highly personalized approaches for immunotherapy. Unlike many solid tumors, hematologic cancers usually carry a low mutation burden and consequently, TSA are predicted to be much less frequent in these neoplasms (72). However, specific B-cell receptor sequence in B-cell malignancies (idiotype) offer an opportunity to specifically target mature B-cell cancers (73). Likewise, the presence of well-characterized fusion proteins in leukemia, notably the BCR-ABL fusion in chronic myelogenous leukemia and acute lymphoid leukemia, enabled the demonstration that circulating T cells could recognize neo-epitopes created by the fusion (74). The infusion of T cells targeting BCR-ABL fusion epitopes in three acute lymphoid leukemia patients bearing the fusion has been associated with molecular remission and trafficking of the antigen-specific T cells to the bone marrow, hence providing a rationale to pursue the development of TSA-based immunotherapy in blood cancers (75).

It is now increasingly recognized that the acquired genetic variants only represent a fraction of the aberrancies leading to an altered MHC-ligandome on cancer cells. Recent evidence shows that transcription and translation of presumed non-coding genetic regions may significantly alter the immunogenicity of malignant cells. These antigens are different from the well-known TAA which originate from canonical reading frames that are either overexpressed and/or abnormally expressed

in neoplastic cells (see below). Therefore, a subclassification for TSA has recently been proposed; mutated TSA (mTSA) and aberrantly expressed TSA (aeTSA) (76). The mTSA derive from mutated DNA sequences in canonical genes that can be either exonic or non-exonic (77, 78) and the aeTSA arise from aberrant and cancer-specific expression of unmutated non-canonical transcripts that are not expressed in normal tissues, including thymic medullary cells (mTECs), which has crucial importance for central tolerance. The aeTSA combine the immunological characteristics of MiHA and mTSA, despite being non-polymorphic and shared between individuals and cancer cells like TAA (76, 79). Importantly, aeTSA that derive from unmutated non-exonic sequences (introns, intergenic regions, etc.) may be very abundant, as revealed by proteogenomic methods in human acute lymphoblastic leukemias and lung cancer samples (76, 80).

Tumor-Associated Antigens (TAA)

Neoplastic cells can overexpress, or aberrantly express, unmutated proteins that are recognized by the immune system (46, 81–83). At present, several TAA have been identified across many cancer types. They are categorized traditionally into four groups: antigens encoded by cancer-gonads genes, embryonic/differentiation genes, overexpressed antigens, and viral antigens. The inclusion of viral antigens as TAA is problematic for several reasons, the most important being that virus-derived antigens are non-self and do not contribute to central tolerance in the thymus like the other TAA. Virus-specific T cells have high functional avidity and have repeatedly been shown to be highly effective for the treatment of Epstein-Barr virus (EBV)-associated lymphoma, particularly in the post-transplant setting (84). Despite issues related to central tolerance, TAA can elicit T-cell responses and TAA-specific T cells can be found at high frequency in the circulating T-cell repertoire of normal individuals (79, 85). Several immunotherapies have been devised to target TAA derived from proteins, such as WT1, NY-ESO-1, PRAME, Proteinase 3, MAGE-A3 in blood cancers and despite inherent limitations, TAA have practical advantages for the design of immunotherapies. The most evident being that being non-polymorphic, they are applicable to a large number of patients and can be prepared using standardized reagents.

MiHA, TSA, TAA, Which Targets to Choose for Blood Cancer Immunotherapy?

The ideal antigenic targets should be highly cancer-specific, be universally applicable to all patients and cancer types and enable treatment without the requirement for AHCT. This last decade has seen the rise of anti-CD19 chimeric antigen receptor (CAR)-modified T cells which fulfill some of these characteristics (86). Despite excellent clinical results in childhood acute lymphoblastic leukemia (ALL), diffuse large B-cell lymphoma (DLBCL) and myeloma, current CAR-based approaches are limited to a subset of B-cell antigens [CD19, CD22 (87), B-cell maturation antigen—BCMA (88)]. This is partly because the on-target/off-tumor reactivity leading to normal B-cell depletion is easy to palliate with exogenous gammaglobulins. Targeting other cells, notably those of the myeloid lineage with CAR therapy may prove to be more difficult as the most promising antigens are also expressed by normal progenitor cells. Finally, toxicities related to this therapy are substantial (89). In fact, the cytokine release syndrome (CRS) and neurological toxicities that follow CAR T-cell infusions require careful patient follow up. The CRS involves fever, hypotension and hypoxia that can rapidly degenerate into organ dysfunction if not treated with anti-cytokine therapy. Likewise, seemingly mild cognitive deficits can rapidly degenerate into encephalopathy and seizure if left untreated. Hence, it is likely that pursuing MHC-associated antigens originating from genetic variations, or variations in the expression of unmutated genetic sequences will offer the promise of immunotherapy for the effective and safe treatment of the full spectrum of blood cancers.

Because they are encoded by germline polymorphisms instead of somatic mutations, MiHA possess features that make them attractive for immunotherapy (6). In contrast to TSA, suitable MiHA are more likely to be expressed by all neoplastic cells and applicable to a large number of patients (71, 90, 91). However, this limitation may not be as important if TSA are derived from shared driver mutations or fusion proteins. For both MiHA and TSA, the use of high avidity T-cell repertoires remains a most appealing element. However, devising and implementing immunotherapies targeting shared epitopes is more convenient. This is a major aspect driving TAA-specific strategies. Lastly, aeTSA may be shared by many tumors, while being non-polymorphic and not inducing central immune tolerance. These characteristics would make aeTSA ideal targets, but much more work is needed in order to evaluate the therapeutic potential of these antigens in humans. The next section will review current and future T-cell therapy strategies (both autologous, and donor-derived in the context of AHCT) directed against these various antigen types.

T-CELL IMMUNOTHERAPY STRATEGIES

The development of methods to identify and characterize MHC-associated antigens resulting from genetic variants is motivated by a strong impetus to design T-cell therapies to treat neoplastic diseases. These T-cell therapies may be used alone or in combination with other approaches, such as vaccination and immune checkpoint blockade but this review focuses on the current status of T-cell therapies aimed at MHC-associated peptides to treat hematological cancers.

T-cell therapies can be antigen agnostic (administered without precise knowledge of the antigens targeted), such as in unmanipulated donor lymphocyte infusions (DLI) and TIL infusion, or targeted to known antigens. The administration of antigen-specific T cells requires prior *ex vivo* manipulations for enrichment and/or expansion of T cells bearing native TCR specific to the targeted antigens. Alternatively, genetic engineering can enable the production of large numbers of TCR transgenic T cells directed against a given antigen.

Antigen Agnostic Approaches

The use of DLI has been one of the most conclusive proof of the GVT effect in AHCT (i.e., objective responses following

the infusion of donor cells without other treatment). However, the efficacy of donor T cells, collected after AHCT and infused in graded doses, has yielded variable results and has a risk of triggering GVHD in 60–70% of patients (92). There is substantial variability in the response rates to DLI based on the underlying disease [from close to 100% in chronic phase chronic myelogenous leukemia to 15–40% in acute leukemia (93)], the disease burden, the timing of administration (pre-emptive vs. advanced disease) and the use of concomitant treatments. Most of the experience in DLI was gained in HLA-matched transplant settings, where MiHA mismatches are the drivers of the alloresponses. With no prior knowledge of the number of antigen mismatches, the tissues in which these MiHA source proteins are expressed and the number of MiHA-specific T cells present in the DLI, this form of immunotherapy does not fully harness the potential of MiHA based immunotherapy in AHCT patients. However, it has the advantage of requiring minimal manipulation and thus be rapidly accessible to a large number of AHCT patients. Other antigen agnostic T-cell therapies have been explored as treatment for solid tumors and blood cancers. An interesting approach is to attempt to exploit T cells harvested from the disease site and reinfuse them after *ex vivo* expansion. TIL therapy was pioneered in solid tumors, in melanoma particularly, where it has yielded high response rates and durable complete remissions (94). This approach is based on the assumption that T-cell populations contained in tumor beds may comprise a high frequency of tumor-reactive cells (95). This principle may also apply for several hematological malignancies. The bone marrow is a natural reservoir of antigen-experienced memory T cells and the site of disease of many blood cancers (96). As such, it may contain a large repertoire of T cells capable of recognizing the malignant hematopoietic cells. Moreover, a practical advantage is that the bone marrow is easily accessible for collection of T cells that can later be expanded *ex vivo*. Expanded autologous "MIL" (marrow infiltrating lymphocytes) from multiple myeloma patients using anti-CD3/CD28 stimulation and IL-2 revealed that the bone marrow contained a high number of myeloma reactive T cells (relative to blood derived T cells from the same patients) capable of targeting both mature and precursor myeloma cells *in vitro* (97). A clinical trial performed in 25 patients confirmed the feasibility of performing "MIL" therapy following autologous stem cell transplant in myeloma patients. The absence of a control group precludes a rigorous assessment of disease response against the standard treatment of this disease, but the authors were able to correlate the presence of anti-myeloma activity in the expanded MILs product, as well as the persistence of anti-myeloma reactivity 1 year after infusion, with favorable outcome (98).

The transfer of a large T-cell repertoire has advantages, such as broad applicability as well as the likelihood of targeting several antigens at the same time. However, antigen agnostic methods can miss the relevant targets by expanding/transferring T cells that are not specific for cancer associated/specific antigens (99). In the setting of AHCT and DLI, this can also lead to toxicity in the form of GVHD. The molecular characterization of MiHA, viral antigens, TAA and TSA now permits the development of more precise and possibly more potent T-cell therapies. This, coupled with more widely accessible T-cell manufacturing methods, allows for the use of manipulated T cells targeting MHC-associated targets in blood cancers.

MHC-Associated Antigen-Specific Approaches in T-Cell Therapy

The current experience using T-cell therapies against MiHA, viral antigens, TSA and TAA demonstrates the possibility to expand antigen-reactive T cells in high numbers to treat patients. However, T-cell manufacturing continues to be challenging and the optimal approach to integrate these therapies in the patients' treatment trajectory remains to be determined. This section reviews the current approaches aiming to treat hematological malignancies through the specific targeting of MHC-associated antigens. A summary of the molecularly defined HLA-associated antigens that have been targeted in adoptive T-cell immunotherapy clinical studies is included in **Table 1** (75, 100–107).

MiHA

As described above, the MiHA have several conceptual advantages for immunotherapy. Vaccination against MiHA

TABLE 1 | MHC-associated antigens targeted in T-cell therapy trials for blood cancers.

Target antigen	Unique or multiple antigen(s)	Antigen type	Natural vs. transgenic TCR	Cancer type	HLA restriction	References
HA-1	Unique antigen	MiHA	Natural	AML, CML, ALL	A0201	(100)
$P2RX7_{265-273}$	Unique antigen	MiHA	Natural	ALL	A2902	(101)
$DPH1_{334-343}$	Unique antigen	MiHA	Natural	MDS	B5701	(101)
DDX37	Unique antigen	MiHA	Natural	ALL	B2705	(101)
BCR-ABL fusion	Antigen library	TSA	Natural	ALL	ND	(75)
$WT\text{-}1_{126-134}$	Unique antigen	TAA	Natural/Transgenic	AML, ALL, MDS	A0201	(102, 103)
$WT\text{-}1_{235-243}$	Unique antigen	TAA	Transgenic	AML, MDS	A2402	(104)
MAGE-A3	Unique antigen	TAA	Transgenic	MM	A01	(105)
NY-ESO-1/LAGE-1	Unique antigen	TAA	Transgenic	MM	A0201	(106)
LMP1, LMP2	Antigen library	Viral Ag	Natural	Lymphoma	ND	(107)

ALL, Acute lymphoblastic leukemia; AML, Acute myeloid leukemia; CML, Chronic myelogenous leukemia; MDS, myelodysplastic syndrome; MM, Multiple myeloma. ND, not defined.

in the context of post-AHCT DLI has been reported to induce detectable MiHA-specific responses in myeloma patients. Although clinical responses were modest (transient regression or stable disease), the vaccination protocol was well-tolerated (108, 109). An alternative approach could be to vaccinate the donors prior to graft collection in order to generate a robust anti-MiHA memory T-cell repertoire in these healthy individuals, as previously done in animal models (55, 110). Unfortunately, this is difficult to envisage for several reasons, including the consequences of allosensitization in donors who may eventually require tissue, cell or organ transplantations themselves.

Cell therapy is the other approach to selectively or preferentially target MiHA. The first trial reporting on a MiHA-specific T-cell therapy strategy used MiHA-specific $CD8^+$ clones obtained by co-culturing donor T cells with host-derived lymphoblastoid cells (EBV-transformed B-cells) (101). After ruling out reactivity to EBV antigens and host fibroblasts (surrogate for non-hematopoietic tissues), reactive T cells were infused. Thus, although highly specific, this approach did not rely on *a priori* knowledge of the targeted MiHA and their tissue distribution. The administration of these T-cell clones led to objective responses in 5/7 refractory relapsing leukemia patients post-AHCT. These responses were short-lived, with evidence of gradual decrease in antigen expression at disease recurrence in at least one patient, hinting at a plausible immune escape mechanism. A surprising complication was the occurrence of pulmonary toxicity, which is not seen following regular DLI. Although, the MiHA source protein could be detected in the lung tissue in one case, the patients had also received a conditioning regimen and post-infusion IL-2, which can be associated with pulmonary complications (111). Nonetheless, these findings are an additional argument to select MiHA with restricted expression to the hematopoietic system. Another trial used donor-derived T-cell lines stimulated *ex vivo* with dendritic cells loaded with the blood lineage and HLA-A0201 restricted MiHA HA-1 (100). Following up to 5 rounds of weekly stimulation with antigen loaded dendritic cells in the presence of IL-2, donor derived T-cell lines containing from 11 to 243 $\times$ 10^6 HA-1 specific $CD8^+$ T cells were infused to 3 relapsing patients post-AHCT. Although clearly demonstrating the feasibility of the approach and its innocuity (no notable GVHD), the procedure was not associated with clinical responses.

In both cases, the advanced disease status of the patients and the prolonged period of T cells in culture can be suspected as limiting factors. It was shown that repeated stimulation with antigen-loaded dendritic cells has a detrimental effect, especially for the targeted MiHA-specific T cells relative to the other T cells present in the culture (112). Upon repeated antigen exposure, the MiHA-specific T cells acquired the expression of PD-1 as well as the terminal differentiation marker KLRG-1, which correlated with their relative failure to expand relative to other T cells in the culture. Other research published in the last decade similarly demonstrated that the acquisition of terminal effector T-cell differentiation and exhaustion features *ex vivo*, compromises the further expansion and persistence of the T cells after adoptive transfer. Less-differentiated T cells bearing early memory T-cell features (central memory—Tcm, or stem cell memory—Tscm) have been shown to be superior compared to more differentiated T cells in several animal and human pre-clinical models (113–115). It was also shown in humans that exposure to T-cell memory differentiating factors early in the culture can program long term persistence *in vivo* despite the expression of effector or effector memory T-cell differentiation markers at the end of the culture (102). The issue of T-cell differentiation is relevant to the whole field of T-cell immunotherapy and the quest for culture conditions that will preserve or promote early memory expression is an active area of research. Candidate pathways and molecules shown to influence memory differentiation include cytokines [IL-21 (102, 116), TGF-β (117)] and metabolic/developmental pathways [AKT (118), WNT (119)].

Gene engineering is a way to avoid the drawbacks of using elaborate and long cultures to expand antigen-specific T cells. The transfer of a transgenic TCR in T cells can be achieved using brief manufacturing protocols that maintain early T-cell memory differentiation and that generate a high number of T cells with the desired antigenic specificity. The efficacy and safety of T cells expressing a transgenic HA-1 specific TCR has been established *in vitro* (120). In this study, an elaborate transgene was used for optimal reactivity and safety. The transgene comprised four elements: a TCR specific to HA-1, a CD8 co-receptor to promote the function of the MHC class I restricted TCR in $CD4^+$ T cells, an inducible caspase 9 safety switch for rapid induction of apoptosis in case of toxicity and a $CD34^-CD20$ tag to facilitate the selection of the cells and to track the cells once transferred (121). This design enabled the expression of the TCR in both $CD4^+$ and $CD8^+$ cells which may contribute to $CD4^+$ T-cell help after transfer. The cells were responsive against different types of primary leukemia cells and cell lines, supporting the further evaluation of HA-1 specific transgenic T cells in clinical trials (NCT03326921). Although the TCR transgenic approach can solve the conundrum of late T-cell differentiation arising in the context of antigen-driven T-cell expansion, it has its own limitations. The production of clinical grade gene therapy vectors is costly and current reports investigating transgenic TCR therapy target only one antigen at a time. TCR transgenic therapies are also limited by the possible mispairing of alpha and beta chain with the endogenous TCR potentially giving rise to unwanted reactivity and toxic allo- or autoimmunity (122, 123). This can be mitigated by the use of murine constant domains, the addition of cysteine residues for preferential pairing of the transgenic chains, α/β chain domain-swapping or the knockdown/out of the endogenous TCR (124–127). However, there is an argument to be made that keeping the endogenous TCR could be beneficial. Chapuis et al. transduced a robust memory EBV-specific T-cell repertoire (which will not cause GVHD) with a TAA-specific TCR transgene in order to leverage the properties of these long term persisting memory cells and use viral reactivations as an adjuvant (103).

Given the possibility for immune escape variants selection following single antigen targeting, the future of MiHA-based therapy may involve multivalent T-cell products (NCT03091933). This emphasizes the importance of discovering and characterizing a large number of MiHA derived from

proteins expressed in the hematopoietic system, as well as MiHA presented by enough HLA alleles to treat most, if not all, AHCT patients.

TSA

The development of T-cell therapies, or vaccines, against TSA or so-called neoantigens is complicated. Identification and validation of neoantigens is time-consuming as well as expensive. The process of preparing vaccines from tissue samples usually takes several months (62, 63). Finally, the development of TSA-specific T-cell immunotherapy may seem unthinkable given the added complexity of T-cell manufacturing. This being said, several approaches can be taken to leverage TSA identification/prediction and design T-cell immunotherapy. Candidate TSA predicted from mutation analysis have been identified using *in vitro* antigen expression system and co-culture with responder autologous TIL (128). Selection and enrichment of these T-cell populations followed by re-expansion represent an attractive strategy to enhance TIL-based, TSA-specific targeting. Interestingly, circulating T cells recognizing neoantigens detected in cancer patients can be found in the peripheral blood of healthy donors (129). In some cases, the cancer naïve repertoire comprises TSA-specific T cells that are not found in the patient TIL which may indicate the loss of certain T-cell clones in cancer patients. Of particular relevance to the development of T-cell therapies, certain mutations within oncogenes occur at the same genetic location, leading to "public" (or shared) T-cell epitopes (130). An example is the G12D *KRAS* gene mutation in digestive cancers, leading to a mutant peptide presented by HLA-C0802 (131). Such "hot-spot" mutations also exist in blood cancers. A recently published study showed that a frequent nucleophosmin 1 mutation in acute myeloid leukemia resulted in the presentation of a neoepitope by HLA-A0201 (132). Finally, given their "public" nature and restricted expression by cancer cells, the aeTSA may represent excellent targets to investigate for the development of T-cell based immunotherapies of hematological malignancies. However, no human studies have been performed to date with aeTSA.

TAA and Viral Antigens

Adoptive T-cell immunotherapy against viral reactivations occurring after AHCT is highly effective, with response rates globally above 70% in otherwise refractory patients (133, 134). In the case of EBV, which is associated with the development of post-transplant lymphoproliferative disorder (PTLD), as well as several lymphoma subtypes outside the context of transplantation, adoptive immunotherapy has a remarkable track record of safety and efficacy (135). Arguably, the prevention or treatment of EBV-associated PTLD after AHCT occurs in the best conditions for T-cell adoptive immunotherapy. The target antigens are foreign, the T cells are expanded (or selected) using multiple antigens from memory T-cell repertoires circulating in immunocompetent healthy donors, who are the original AHCT donors or even partially HLA-matched third-party donors. The resulting T-cell products are polyclonal, can display reactivity against antigens bound by several HLA alleles, usually contain both EBV reactive $CD4^+$ and $CD8^+$ T cells and, depending on the manufacturing protocol, express early memory T-cell markers. The use of peptide libraries containing multiple epitopes derived from several antigenic EBV proteins (such as LMP2, EBNA1, and BZLF1) allow the generation of multivalent T-cell products (136). Virus-specific T-cell lines are effective after AHCT or even solid organ transplant and can be used as prophylaxis in patients at high risk of PTLD with excellent result and no significant GVHD or organ rejection (137–139). The mobilization of the autologous EBV T-cell repertoire in previously treated lymphopenic lymphoma patients outside the context of transplantation requires more elaborate *ex vivo* culture protocols, but is nonetheless feasible and well-tolerated (107). Bollard et al. reported on 29 patients with EBV-associated lymphoma who received the T-cell lines as consolidation following the achievement of remission (one relapse after a median follow up of 3.1 years) and 21 patients who had active disease at the time of infusion. Among these, 13 had clinical responses (11 complete responses) with evidence of T-cell reactivity against the targeted EBV antigens (LMP1, LMP2) and TAA, evoking the possibility of epitope spreading.

Expanding on the success of anti-viral therapy, it was shown that T-cell lines can be generated by stimulating with overlapping peptide libraries of multiple TAA (140). These T-cell lines products were reactive to multiple TAA simultaneously, were polyclonal, displayed early memory T-cell markers and could be generated from both healthy donors and lymphoma patients. Trials are currently testing the clinical effects of such multivalent TAA-targeting T-cell lines in several blood cancer types (NCT02203903, NCT02494167, NCT02475707, NCT02291848, NCT01333046). Because TAA are molecularly defined and non-polymorphic, they are more easily amendable to transgenic TCR therapy. The isolation and cloning of TAA specific TCR restricted by common HLA alleles can yield TCR sequences that can be used in a large population of patients. For the same reasons, TAA have been used in vaccine trials in the setting of various blood cancers including multiple myeloma, lymphoma, and acute myeloid leukemia [reviewed in Avigan and Rosenblatt (141)]. Transgenic TCR therapy against TAA expressed by hematopoietic cancers was also tested in several clinical trials. Transgenic MHC class I restricted TCR against NY-ESO-1/LAGE-1 and MAGE-A3 have been used to treat myeloma patients. In both cases, the TCR were engineered for increased affinity for the MHC-peptide complex as a way to circumvent a limitation of TAA-based immunotherapy as described above. The use of autologous engineered NY-ESO-1 specific T cells administered in the context of autologous transplantation resulted in clinical responses in 16/20 patients (106). The adoptively transferred T cells showed expansion as well as trafficking to the bone marrow, and did not cause significant toxicity. Expectedly, loss of antigen or lack of persistence of the transferred T cells were associated with relapse. In the case of MAGE-A3, enhanced affinity TCR transgenic T cells caused unexpected and rapid cardiotoxicity in the first 2 patients recruited on the trial (105). Cross-reactivity with a peptide derived from the heart muscle protein TITIN was the causative mechanism. These trials showed both the

promise and perils of using affinity enhanced TCR in cancer adoptive immunotherapy. Native and unaltered TAA-specific TCR gene transfer has also been performed. The transcription factor WT1 is overexpressed in several blood cancers and contributes to several known MHC class I associated epitopes. A first trial involving transgenic WT1 specific TCR has been reported in 2017. The study was performed in patients suffering from refractory acute myelogenous leukemia and high risk myelodysplastic syndromes (104). The treatment involved the administration of two T-cell infusions and post-transfer WT1 vaccination. Eight patients were treated in two dose groups. Two objective, but transient, responses were noted and among the five patients who had persisting circulating engineered T cells, four survived more than 12 months. No significant toxicity was observed. More recently, another study was reported using a different transgenic native (but selected for high affinity) TCR against an HLA-A0201 restricted WT1 peptide and transduced in EBV-specific memory T cells (103). The cells were administered to prevent acute myeloid leukemia relapse after AHCT, when the disease burden is low. With a relapse free survival of 100% at a median of 44 months of follow up (compared to 54% in a concurrent control group), an argument can be made about the importance of administering T-cell therapy early in the treatment trajectory of patients.

PERSPECTIVES AND CLINICAL INTEGRATION OF T-CELL THERAPIES

The opportunities for antigen-specific T-cell immunotherapies are rapidly expanding. The MHC-associated antigens arising from genetic variants, both germline and acquired through the neoplastic process, are prime targets for the treatment of hematological cancers. The genuinely personalized approaches required to translate the complexity and multiplicity of MiHA, TSA, and TAA into therapy is certainly a challenge, but also a great promise. The discovery and characterization of an increasing number of antigens will enable the design of multivalent therapies capable to target all blood cancers and limit the emergence of immune escape variants associated with single antigen targeting. However, for such promise to materialize, manufacturing processes for these highly personalized therapies will have to be refined and made cost-effective. Nonetheless, T-cell therapies aimed at MHC-associated peptides have the potential to significantly expand existing paradigms in AHCT, autologous cell transfer and other T-cell therapies, such as CAR T cells. Indeed, the development of peptide-MHC specific antibodies may further increase the relevance of characterizing immunogenic MiHA, TAA or shared TSA for CAR-based immunotherapy (142, 143). Along the same lines, genetic variants may also create non-MHC associated cell surface epitopes targetable through recognition by antibodies. Finally, existing CAR may be transduced and expressed in antigen-specific T cells recognizing viral, TAA, MiHA, or TSA through their natural TCR and thus enable dual targeting of malignant cells. The development of multivalent T-cell products, either as a combination of T cells specific for a single antigen or T cells with multiple specificities, will be essential to avoid the emergence of immune escape variants following therapy. In addition, approaches aimed at targeting multiple antigens may prove to be synergistic. For example, pre-clinical studies have shown that only a combination of T cells targeting Y-chromosome derived MiHA and TAA could lead to tumor regression. A threshold effect may be required to generate enough inflammation to support effective anti-cancer immunity (144, 145). Similarly, this is likely achieved in AHCT settings by Y-chromosome antigen-specific T cells given the increased GVT and GVHD effects noted after female into male transplants (146). Another possible benefit of inducing strong immune responses is the development of epitope spreading as evoked by the appearance of detectable anti-TAA responses following microtransplantation, AHCT or anti-viral T-cell therapy (147, 148). Along the same lines, the combination of T-cell therapy with other immunotherapeutic interventions is also likely to unveil important synergies. To this end, the administration of vaccines to consolidate the response after adoptive transfer, or immune checkpoint inhibitor therapy following adoptive T-cell infusion, are actively investigated.

The timing of administration of T-cell therapies will need to be better studied (**Figure 3**). Cell therapies remain largely offered to refractory patients. However, the promising results following prophylactic DLI (149, 150), anti-viral T-cell lines (137) and more recently transgenic TCR therapy (103), suggest that T-cell therapies should not be confined to the treatment of relapsing patients. In fact, these treatments are probably more potent in the context of low burden disease. The reassuring safety profile of several of the approaches targeting MHC-associated peptides should facilitate the introduction of T-cell therapies earlier on during the patient's course.

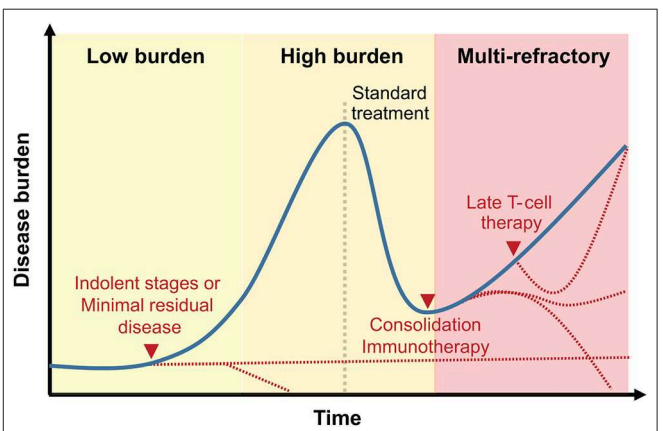

FIGURE 3 | Clinical integration of T-cell therapies targeting MHC-associated antigens. Representation of T-cell therapy timing relative to disease history. While early treatment or treatment following a reduction in disease burden may be associated with prolonged remission (dotted red lines), late-stage blood cancers treatment with MHC-associated antigen-specific T cells may only delay disease progression.

Several scientific and methodological issues remain to be addressed to improve T-cell therapies directed against MHC-associated peptides or the MHC molecule itself. A significant contributor to the response against genetic variants is $CD4^+$ T-cell mediated, but most identified MiHA, TAA and TSA are MHC class I associated peptides (151). Although challenging, the identification of MHC class II restricted responses will likely be essential to optimize T-cell therapies. This should be a major area of research in the upcoming years.

The downregulation or loss of MHC expression, the genetic loss or silencing of antigen source protein are well-known immune escape mechanisms in cancer. This can be fairly extensive as described in haplo-identical transplants, where the loss of the entire mismatched haplotype can be observed (152). Elaborate strategies targeting MHC-associated peptides presented by different alleles and belonging to different haplotypes may be necessary to harness the therapeutic potential of T-cell immunotherapy against genetic variants translated into MHC-associated peptides. Moreover, an attractive combination approach is to maximize antigen presentation though epigenetic modulation. Demethylating agents, histone deacetylase inhibitors and methyltransferase inhibitors are established or investigational drugs for the treatment of blood cancers. It is increasingly recognized that these also promote gene expression that increases the immunogenicity of malignant cells and also affect immune cell physiology [reviewed in Lindblad et al. (153)]. These effects have been reported to occur through multiple mechanisms like cytokine expression, as well as upregulation of the MHC and associated antigens (154, 155). This last aspect is particularly intriguing as both TAA and cryptic aeTSA antigens have been shown to be promoted by epigenetic modulation (156–159). Notably, extra-exomic endogenous retroviral elements which are attractive as a source of specific and robust cancer antigens can be expressed through modulation of methylation. However, as a note of caution, there is conflicting reports on the outcome of epigenetic modifiers on the physiology of immune cells. Among others, regulatory T cells and the expression of immune checkpoints can be promoted by these agents, perhaps inviting for further combinations with immune modulators.

To conclude, the field is increasingly confronted with multiple antigens and approaches to target them. Careful selection of the best targets will need more research and rational combinations therapies are likely to be required for these antigens to reveal their full potential.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. Specifically, VJ, CR, and J-SD contributed to the manuscript design. VJ, CR, SD, CC, and J-SD performed the literature search and contributed to the writing of the manuscript.

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MINI REVIEW
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TCR-Like CAR-T Cells Targeting MHC-Bound Minor Histocompatibility Antigens

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Minor histocompatibility antigens (mHAgs) in allogeneic hematopoietic stem cell transplantation are highly immunogenic as they are foreign antigens and cause polymorphism between donors and recipients. Adoptive cell therapy with mHAg-specific T cells may be an effective option for therapy against recurring hematological malignancies following transplantation. Genetically modified T cells with T cell receptors (TCRs) specific to mHAgs have been developed, but formation of mispaired chimeric TCRs between endogenous and exogenous TCR chains may compromise their function. An alternative approach is the development of chimeric antigen receptor (CAR)–T cells with TCR-like specificity whose CAR transmembrane and intracellular domains do not compete with endogenous TCR for CD3 complexes and transmit their own activation signals. However, it has been shown that the recognition of low-density antigens by high-affinity CAR-T cells has poor sensitivity and specificity. This mini review focuses on the potential for and limitations of TCR-like CAR-T cells in targeting human leukocyte antigen–bound peptide antigens, based on their recognition mechanisms and their application in targeting mHAgs.

Keywords: minor histocompatibility antigen, TCR-like antibody, adoptive immunotherapy, allogeneic stem cell transplantation, chimeric antigen receptor (CAR) cell

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INTRODUCTION

Minor histocompatibility antigens (mHAgs), which are generated from polymorphic genes between a donor and recipient, are presented in the groove of human leukocyte antigen (HLA) molecules. In recipients undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT), mHAgs are recognized by donor T cells (1) and are highly immunogenic in the graft-vs.-host direction (2). Detection of T cell responses to molecularly defined and well-characterized mHAgs following allo-HSCT is possible through use of an HLA multimer reagent that incorporates the defined epitope peptide (3). In the context of hematologic malignancies, the therapeutic potential of T cells specific to mHAgs presented predominantly or exclusively on recipient target hematopoietic cells (including leukemia cells) but not on non-target non-hematopoietic cells has been shown via the graft-vs.-leukemia effect following donor lymphocyte infusion against recurring hematological malignancies (2–4). In addition, some mHAgs such as HA-1 and BCL2A1 have been found expressed in solid tumors, supporting the clinical applicability of immunotherapy in the allo-HSCT setting (5, 6). However, it is not always possible to selectively expand mHAg-specific T cells for their use in adoptive immunotherapy, primarily because of the cumbersome and time-consuming *in vitro* expansion procedure, which sometimes results in T cell exhaustion (7, 8). To overcome

this problem, viral vectors encoding T cell receptor (TCR) α and β chain cDNAs cloned from high affinity mHAg-specific T cells have been used to genetically modify and redirect T cells toward the targeted mHAg (9, 10). Indeed, these so-called "TCR-T" cells have been shown to acquire the conferred antigen specificity, but mispairing between the introduced and endogenous TCR chains occasionally results in unwanted or unpredictable T cell specificities (11). Competition for CD3 complexes also leads to decreased signal transduction necessary for T cell function and proliferation. Various countermeasures have been devised to address these problems, including (1) the use of constant domains from other species such as mice (12), (2) introduction of disulfide or other bonds between the α and β TCR chains (13), (3) silencing of endogenous mRNA encoding TCR using siRNA (14), and (4) knockout of the TCR gene by means of gene editing technologies (15). An alternative approach was the development of chimeric antigen receptor (CAR)–T cells with TCR-like specificity, whose transmembrane and intracellular domains do not compete with endogenous TCR for CD3 complexes. This mini review will focus on the potential and limitations of applying TCR-like CAR-T cell technology to target HLA-bound mHAgs.

BACKGROUND OF TCR-LIKE ANTIBODIES AND THEIR CAR-T FORM

Recently, CD19-specific CAR-T cell therapies have been introduced in clinical practice with great success. Although clinical trials of CAR-T cells targeting promising candidate antigens other than CD19 are underway, the number of ideal tumor-specific targets is limited by the number of tumor-specific "cell-surface" antigens that are targetable with conventional monoclonal antibodies. By contrast, most potential tumor-specific antigens, such as proteins involved in cell proliferation and survival, are located in the intracellular region; there, they are degraded by proteasomes and may be displayed as antigenic peptides on major histocompatibility complex (MHC) class I and MHC class II molecules. These MHC-bound antigens are recognized by T cells with specific TCRs under physiological conditions. The affinity of the relevant TCRs is generally moderate or low because most tumor antigens are shared with normal cells but are generally overexpressed. Thus, reactive T cells with high-affinity TCRs undergo negative selection in the thymus (16). To target MHC-bound antigens, enhancement of TCR affinity with amino acid substitutions or development of a new mode of antibodies specific for peptide/MHC (pMHC) complexes is necessary. The latter are called TCR-like or TCR-mimic antibodies and can be used to redirect T cells to target antigens. The first TCR-like antibody was developed in 1982 to target the influenza PR8 antigen presented on murine H-2K^b (17), and the first attempt to construct CAR-T cells with a TCR-like antibody against MAGE-A1 presented on HLA-A1 was conducted in 2001 (18).

Initially, to generate TCR-like antibodies, a hybridoma-based method was used in which animals (mostly mice) were immunized with cells expressing pMHC or recombinant pMHC proteins. Then, sensitized splenic B cells were isolated and fused with a non-secretory myeloma cell line, which resulted in the generation of hybridoma cells, each of which produced monoclonal antibodies. New technology able to synthesize recombinant pMHC monomers or multimers (19) contributed to the preparation of antigens required not only for immunization, but also for the screening of hybridoma libraries. Antibodies raised by hybridoma-based methods consist of naturally selected light and heavy chain pairs; they possess a high affinity but have a limited repertoire of diversity because the pool size is restricted by the number of initial splenic B cells in the immunized mice.

In contrast, new phage library-based methods (20) utilize phages carrying randomly combined variable regions from light and heavy chains that have been amplified from a B cell pool. Their diversity size is approximately 10^9–10^{10}. It is possible to screen phages by positive and negative selection with target antigens and non-target antigens under various stringency conditions, as the fused variable genes in the recombinant phage genome are displayed as single-chain antibodies on their phage surface. Because most procedures can be performed with biochemical assays, this approach is robust and cost-/time-effective. However, the random recombination of variable regions from irrelevant light and heavy chains sometimes leads to antibodies with off-target binding capacity in addition to the desired pMHC specificity. Thus, careful and thorough screening in a wide array of normal tissues is necessary.

Nearly half of the reported TCR-like antibodies have been generated by the phage-based method (21). Among these, only 11 reports, including ours (22), described the application of TCR-like antibodies to CAR-T cell development (18, 23–32). As shown in **Table 1**, 10 of 12 such CAR-T cells targeted non-mutated antigens highly expressed in tumor cells, but none of them have been evaluated in clinical trials so far. All but one study that targeted the insulin-derived peptide presented on MHC class II used phage libraries whose clone size ranged from 2.85×10^8 to 9×10^{10} clones. The dissociation equilibrium constant (K_D) of their binding moiety ranged widely from 0.03 to 400 nM. This is in marked contrast to TCRs, the affinities of which generally range between 1 and 100 μM (33). Thus, natural TCR affinity is approximately 10^3 to 10^5 times weaker than those of the TCR-like antibodies. In addition, it has been shown that the use of the antibody's binding moiety as the antigen recognition domain of CARs can increase effector function, leading to the eradication of tumor cells with downregulated antigen expression at a level of 200 copies/cell (34). A similar density threshold of 300 copies/cell was reported for murine CAR-T cells targeting the glycoprotein OTS8 induced by cancer-specific mutation (35). As mentioned earlier, TCR gene-modified T (TCR-T) cells have also been developed since the first attempt by Heemskerk et al. (9). One phase I trial for TCR-T cells targeting HA-1 mHAg is currently underway (NCT03326921). The similarities and differences among TCR-like CAR-T cells, TCR-T cells, and conventional CAR-T cells are shown in **Figure 1**.

TABLE 1 | List of TCR-like CAR-T cells.

Antigen	MHC	Epitope	Target disease	Method	Library size	Immunization	Screening	Clone	Affinity (K_D)	CAR signaling domains	References
MAGE-A1	HLA-A1	EADPTGHSY	Cancer	Phage	3.7×10^{10}	–	pMHC	G8	250 nM	CD4–FcεRIγ	(18)
MAGE-A1	HLA-A1	EADPTGHSY	Cancer	Phage	3.7×10^{10}	–	pMHC	G8 Hyb3	250 nM 14 nM	CD28–FcεRIγ	(23)
NY-ESO-1	HLA-A*02:01	SLLMWITQC	Cancer	Phage	1.45×10^{10}	–	pMHC	T1	2–4 nM	CD28–CD3ζ	(24)
WT1	HLA-A*02:01	RMFPNAPYL	Cancer	Phage	3.7×10^{10}	–	pMHC	F2 F3	400 nM 30 nM	CD28- FcγRIγ	(25)
HMHA1	HLA-A*02:01	VLHDDLLEA	Blood cancer	Phage	5×10^{8}	+	pMHC	#131	19.9 nM	CD28–CD3ζ	(22)
GP100	HLA-A*02:01	ITDQVPFSV	Melanoma	Phage	5.4×10^{8}	–	pMHC	GPA7	183 nM	CD3ζ	(26)
WT1	HLA-A*02:01	RMFPNAPYL	Cancer	Phage	2.85×10^{8}	–	pMHC	Q2L	3 nM	CD137–CD3ζ	(27)
AFP	HLA-A*02:01	FMNKFIYEI	Hepatoma	Phage	9×10^{10}	–	pMHC	ET1402L1	0.03–0.2 nM	CD28–CD3ζ	(29)
WT1	HLA-A*02:01	RMFPNAPYL	Cancer	Phage	7×10^{10}	–	pMHC	ESK1	0.1 nM	CD28–CD3ζ	(30)
WT1	HLA-A*24:02	CYTWNQMNL	Cancer	Phage	NA	–	pMHC	#213	741 nM	CD3ζ-CD357	(31)
Insulin	H-2 I-A^{g7}	B:9–23 peptide	Autoimmune diabetes	Hybridoma	850	+	pMHC	mAb287	120 nM	CD28–CD3ζ, CD28–CD137–CD3ζ	(32)

AFFINITY OF TCR-LIKE ANTIBODY AND DENSITY OF TARGET pMHC

While the target antigen density per tumor cell in antibody-targetable tumors has not been measured in detail, the proteins CD19 and CD20 present on B cells targeted by therapeutic antibodies and CAR-T cells are relatively well studied; for example, the antigen density of CD19 has been reported to be 2×10^4 to 3×10^4 molecules/cell in normal B cells and 0.5×10^4 to 3×10^4 molecules/cell in malignant B cells (36, 37). In contrast, despite the fact that the affinities of canonical native TCRs are 3 to 5 logs lower than those of the conventional antibodies used in CD19 CAR-T cells, it is interesting that T cells can recognize pMHCs presented on cell surfaces at much lower densities. Several studies have demonstrated that the minimum number of pMHC complexes required to activate T cells is <10 per cell, although this number depends on the presence of coreceptors and the status of the cell (38–40). Using comprehensive mass spectrometry analysis of a peptide pool stripped from MHC molecules, it has been shown that certain peptides are expressed at a frequency of 100 to 10,000 copies/cell, which corresponds to 0.1 to 10% of peptides presented by one kind of MHC allele, such as H-2 K^b or D^b in mice (38). Assuming the number of each MHC class I molecule per cell is 1×10^5 to 2×10^5 and the average copy number of a given peptide is 200, then 500–1,000 peptides with different sequences are expressed on one kind of MHC per cell (38). However, peptides with much lower densities must be expressed at a much wider variety and still be recognized by relevant T cells.

In terms of human mHAgs, it has been shown that HLA-A*02:01–restricted HA-1^H is present at 80 copies/cell, while its counterpart HA-1^R is <5 copies/cell because of its 27-fold lower affinity to the HLA-A2 molecule (41, 42). The other HLA-A*02:01–restricted HA-2^M mHAg was found to be present below the detection limit of 0.04 to 0.2 copy/cell (43). It is speculated that these cytotoxic T lymphocytes (CTLs) possess high-affinity TCRs because mHAgs are non–self-antigens similar to pathogens (44), and thus no thymic or peripheral tolerances affect T cells (16). Given that CTLs specific for HA-1^H and HA-2^M were readily detected at a range of 0.21 to 1.57% among $CD8^+$ cells in patients receiving allogeneic HSCT and donor lymphocyte infusion and that the sorted T cells showed specific killing activity against mHAg-positive target cells (3), it is clear that T cells should have at least two modes of action when recognizing antigens via canonical (cognate) TCR moieties vs. CAR moieties.

A small number of pMHC complexes can serially engage and trigger up to approximately 200 TCRs (45). Additionally, efficient T cell activation occurs within an optimal dwell-time range of TCR–pMHC interaction using MHC with mutations in its antigen-binding site (46). This is thought to be possible by a TCR–pMHC engagement of moderate affinity rather than super-high affinity as seen in antibody–antigen binding. Furthermore, it has been shown that CD20 CAR-T cells require approximately 15,000 CD20 molecules per target cell to trigger 10,000 CAR molecules per T cell, suggesting that a decreased number of triggered CAR molecules are necessary because

	TCR-T	TCR-like CAR-T	Conventional CAR-T
Receptor	T-cell receptor heterodimer	Single-chain variable fragment (scFv) from antibody	Single-chain variable fragment (scFv) from antibody
Target antigen	Peptide/MHC complex (intracellular protein)	Peptide/MHC complex (intracellular protein)	Cell surface antigen
MHC restriction	Dependent	Dependent	Independent
Minimal number of antigen per cell	1	Not fully studied, but 100<	100<
Range of receptor affinity (Kd)	$10^{-4} \sim 10^{-6}$ M	$10^{-6} \sim 10^{-9}$ M	$10^{-6} \sim 10^{-9}$ M
Costimulatory molecules	CD28, CD137	Linked directly to scFv (CD28 and/or CD137 in combination with CD3ζ)	Linked directly to scFv (CD28 and/or CD137 in combination with CD3ζ)
Corecptors	CD4 for MHC-II, CD8 for MHC-I	Unknown, some involvement of CD8 for MHC-I	Not fully studied
Serial killing function	Yes	Yes	Yes
Administration	One infusion	One infusion	Once infusion
Challenges	Cell manufacturing, competition to endogenouse TCR	Cell manufacturing	Cell manufacturing

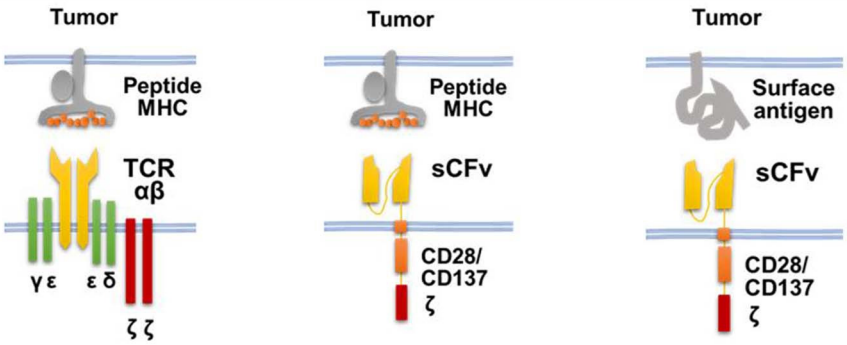

FIGURE 1 | Characteristics of TCR-T and TCR-like CAR-T cells.

of a lack of serial engagement (47). In contrast, decreased signaling and effector function did not occur when high-density antigens were present on the target cells (48). These observations shed light on the design of CAR-T cells equipped with TCR-like antibodies.

CONSIDERATIONS TOWARD CAR-T CELLS EQUIPPED WITH TCR-LIKE ANTIBODIES

Attempts to generate CAR-T cells possessing a TCR-like antibody moiety (TCR-like CAR-T) have been challenging, insofar as target cells express a very low density of pMHC. Furthermore, it has not been clarified whether a "serial engagement" scenario can occur even in the case of TCR-like CAR-T cells with a TCR-like antibody moiety that has low affinity comparable to canonical TCR. To this end, fine tuning of the TCR-like antibody moiety is crucial. Crystal structural analysis revealed that TCRs bind in a conserved diagonal mode (33); thus, some guidelines for tuning their affinity either to the epitope peptide or to an MHC scaffold have been devised. Alternatively, TCR-like antibodies take various binding modes, and their fine tuning is limited to the complementary determining region 3 (49, 50).

Researchers have attempted to ensure the specificity of modified antibodies in targeting amino acids among an array of peptides presented on a single restriction MHC molecule. This is critical because expression as a CAR-T form on the T cell surface, where other adhesion molecules and coreceptors are aligned, may further modify the functional avidity of CAR-T cells. Akahori et al. (31) comprehensively analyzed their low-affinity TCR-like CAR-T cells specific to the $WT1_{235-243}$ peptide presented on HLA-A*24:02 molecules by incorporating (1) alanine substitution analysis of the epitope peptide to identify both the amino acid residues that trigger interaction with TCR CAR-T cells and those used for anchors; (2) *in silico* searches for potentially cross-reactive peptides that contain the predetermined contact amino acid residues in their sequence, followed by *in vitro* assays to test their potential to stimulate TCR CAR-T cells; and (3) *in vitro* cross-reactivity assays against other HLA molecules using a panel of cell lines. Their TCR-like

antibody (clone #213) has a K_D of 741 nM (31), which is close to the lowest natural TCR affinity range of 1 to 100 μM (33). This strategy may contribute to the sufficient functional avidity (here, a biological readout reflecting T cell responsiveness *in vitro*) and retained specificity of their TCR-like CAR-T cells, although the $WT1_{235-243}$ peptide density on WT1- and HLA-A24–positive cells has not been determined to date. It has been shown that two kinds of conventional CAR-T cells, with K_D values of 1 nM and 1,616 nM to the same extracellular domain of HER2 molecule, had comparable lytic activity against target cells with high HER2 expression; however, CAR-T cells with low affinity showed more efficient lytic activity against target cells with limited HER2 expression (51).

Of additional concern are on-target/off-tumor and off-target toxicities. Such toxicities have been observed in adoptive immunotherapy trials using affinity matured TCR-T cells specific to MAGEA3 (52) or CAR-T cells specific to CA9 (53) or CEA (54), all of which are expressed in normal tissues at very low levels. Oren et al. (25) demonstrated that their TCR-like CAR, which had an elevated receptor affinity (30 nM) compared with that of others (**Table 1**), results in some loss of specificity and decreased cell survival when transduced into HLA-A2–positive but HLA-A2–negative T cells. This may be due to fratricide, wherein a high-affinity antibody cross-reacts with non-target peptides presented on coexisting T cells. A similar phenomenon has been reported in which the addition of an anti-CD38–blocking antibody saved CD38 antibody-equipped CAR-T cells from fratricide, as CD38 is dimly expressed on T cells (55). To prevent these toxicities in future clinical studies, a systematic screening system for cross-reactivity testing must be devised. A humanized mouse model, where HLA-matched tumor cells and immune cells from the patient are engrafted into an HLA-transgenic non-obese diabetic/severe combined immune-deficient/common gamma chain knockout mouse, may serve as a screening platform (56, 57).

APPLICATION OF TCR-LIKE CAR-T CELLS TO MHAGS

Inaguma et al. (22) first included a TCR-like antibody against the HA-1^H mHAg in CAR-T cell preparations (**Table 1**). Using a phage library prepared from splenic B cells isolated from HLA-A2–transgenic mice immunized with HA-1^H/HLA-A*02:01 tetramers, specific single-chain antibodies were isolated by multiple rounds of panning. HLA-A2 transgenic mice were used to omit xenogeneic immune responses against human HLA-A2 molecules. Although a resulting CAR-T cell (clone #131) with high affinity binding (K_D = 19.9 nM) was stained with HLA-A2/HA-1 tetramers with an intensity equivalent to cognate cytotoxic T cell clones, the CAR-T cell required 100-fold higher peptide density to exert cytotoxic function (22). Another clone (#9) with moderate to low affinity (K_D = 446 nM) was also tested, and researchers found that its CAR-T form exhibits ∼10-fold increased activity, supporting the observations by Akahori et al. (31).

Major histocompatibility complex class I–restricted autosomal mHAgs are generated by various molecular mechanisms (2, 58). The majority of mHAgs are generated by single-nucleotide substitutions that engender amino acid substitutions, whereas others are generated by frameshift mutations or whole gene deletions (e.g., UGT2B17) (59). Among these, allelic variant peptides of mHAgs such as ACC-1 (60, 61) and HB-1 (62, 63) are expressed with an affinity similar to that of their restriction HLA molecules. Generating TCR-like antibodies to these mHAgs may be difficult, as the difference between two allelic variants is a single amino acid, and TCR-like antibodies may bind to more than one (e.g., three) amino acid in the peptide (31). Given this issue of specificity, it is more reasonable to target mHAgs in which only one allelic variant is exclusively or at least highly expressed. Because the HA-1^R peptide cannot be presented on the cell surface (42), HA-1^H is an ideal target. Other mHAgs resulting from frameshift mutations due to various polymorphisms, such as LRH-1 (64), HMSD (65), or PANE1 (66), may be suitable, as the donor-recipient pair possesses different amino acid sequences at the corresponding positions (or a null peptide in the case of gene deletion or miss-sense polymorphisms). Finally, target mHAgs must be hematopoietic system–specific to avoid graft-vs.-host disease.

In the production of antibodies, both major methods (hybridoma and phage library) have advantages and disadvantages. Although it is thought that naturally occurring antibodies with TCR-like specificity are extremely rare, with the exception of those against mHAgs encoded on the Y chromosome (H-Y antigen) (67), such antibodies can be present in patients receiving mHAg-mismatched HSCT. If this is the case, pooled B cells from such patients may serve as a source for phage display library. Alternatively, immunization with mHAg-pMHC complexes from HLA-transgenic mice (22) may also be a source.

CONCLUSION

Based on the state of the field, the generation of TCR-like CAR-T cells that use an all-in-one chimeric receptor equipped with modifiable intracellular signaling domains that can be applied quickly to patients as adoptive cell therapy is of utmost interest. Chimeric antigen receptor–T cells have a strong advantage over modified TCR gene–introduced T cells because they have a built-in signaling domain, which works even in the absence or downregulation of costimulatory signals from target cells. However, various improvements in TCR gene–introduced T cells make this option safer and more promising than are CAR-T treatments (10). In any case, the establishment of robust and efficient screening systems, including a variety of panel peptides, HLA-typed cell lines, and animal models for the evaluation of TCR-like antibody efficacy and toxicity, as well as the careful planning of preclinical experiments, is necessary for obtaining TCR-like antibodies with potential clinical applications. In

contrast to passive immunotherapies such as gene-modified T cells, active immunotherapies such as peptide (68, 69) or DNA vaccinations with or without adjuvants using dendritic cells are being tested in clinical trials (2). These approaches are thought to be more feasible and less expensive, as gene-modified cells are under strict regulations. However, it is too early to compare the two major approaches as only limited phase I/II clinical data have been publicly reported, including a phase I dose evaluation study for an HA-1 mHAg vaccine (70). Further studies for individual interventions are necessary to define the optimal methods and patient populations for mHAg-targeted immunotherapy.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Discovery and Differential Processing of HLA Class II-Restricted Minor Histocompatibility Antigen LB-PIP4K2A-1S and Its Allelic Variant by Asparagine Endopeptidase

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Minor histocompatibility antigens are the main targets of donor-derived T-cells after allogeneic stem cell transplantation. Identification of these antigens and understanding their biology are a key requisite for more insight into how graft vs. leukemia effect and graft vs. host disease could be separated. We here identified four new HLA class II-restricted minor histocompatibility antigens using whole genome association scanning. For one of the new antigens, i.e., LB-PIP4K2A-1S, we measured strong T-cell recognition of the donor variant PIP4K2A-1N when pulsed as exogenous peptide, while the endogenously expressed variant in donor EBV-B cells was not recognized. We showed that lack of T-cell recognition was caused by intracellular cleavage by a protease named asparagine endopeptidase (AEP). Furthermore, microarray gene expression analysis showed that PIP4K2A and AEP are both ubiquitously expressed in a wide variety of healthy tissues, but that expression levels of AEP were lower in primary acute myeloid leukemia (AML). In line with that, we confirmed low activity of AEP in AML cells and demonstrated that HLA-DRB1*03:01 positive primary AML expressing LB-PIP4K2A-1S or its donor variant PIP4K2A-1N were both recognized by specific T-cells. In conclusion, LB-PIP4K2A-1S not only represents a novel minor histocompatibility antigen but also provides evidence that donor T-cells after allogeneic stem cell transplantation can target the autologous allelic variant as leukemia-associated antigen. Furthermore, it demonstrates that endopeptidases can play a role in cell type-specific intracellular processing and presentation of HLA class II-restricted antigens, which may be explored in future immunotherapy of AML.

Keywords: minor histocompatibility antigens, CD4 T-cells, HLA class II, allogeneic stem cell transplantation, graft vs. leukemia effect

INTRODUCTION

HLA-matched allogeneic stem cell transplantation (alloSCT) is a routinely applied treatment option for many hematological malignancies (1). Donor-derived T-lymphocytes can thereby recognize residual malignant cells of the patient, leading to the beneficial graft vs. leukemia (GvL) effect (2). These T-cells are directed against minor histocompatibility antigens, which are polymorphic peptides differentially presented on patient and donor cells that are able to elicit $CD8^+$ or $CD4^+$ donor T-cells in the context of self-HLA (3). A variety of HLA-class I- and II-restricted minor histocompatibility antigens have been identified by different techniques (4–11), and for several of these antigens, the appearance of specific $CD8^+$ T-cells was closely followed by complete remissions of the malignancies (12–15), indicating the clinical relevance of these T-cells.

Different mechanisms have been shown to create HLA class I-restricted minor histocompatibility antigens. Most antigens are encoded by "missense" single-nucleotide polymorphisms (SNPs) in coding gene regions that directly lead to an amino acid change in the protein. Antigens can also be created by SNPs in coding gene regions that are synonymous in the normal reading frame, but missense in an alternative reading frame. In addition, antigens can be derived from proteins that are translated in an alternative reading frame as a result of small indels, such as for LRH-1 (13) or due to the presence of a non-polymorphic alternative start codon as shown for LB-ECGF-1H (14) and LB-ADIR-1F (15). Even SNPs in non-coding gene regions can create polymorphic antigens as a result of alternative mRNA splicing, as reported for PANE1 (16), ACC-6 (17), ITGB2 (18), and TTK (19). Finally, antigens can be encoded by polymorphic genes that are present in the patient, but homozygously deleted in the donor as shown for UGT2B17 (20).

Besides a direct difference in interaction of the presented peptide with the T-cell receptor (TCR), also differential processing of patient and donor variants can determine the immunogenicity of a polymorphism. Intracellular processing has been described to preclude surface presentation of the donor variant of HA-3 (21), HA-8 (22), LB-NUP133-1R (23), and possibly HA-2 (24). These donor variants induce strong T-cell activation after exogenous peptide loading, but fail to activate specific T-cells when endogenously expressed, demonstrating that insufficient processing of the donor variant into the HLA class I pathway is the underlying reason for lack of T-cell recognition. Finally, it has been shown for HA-1 that impaired binding affinity of the donor variant to HLA-A*02:01 leads to an increased dissociation and therefore insufficient surface presentation (25).

Here, we present the identification of four new HLA class II-restricted minor histocompatibility antigens by whole genome association scanning (WGAS). For three of these antigens, donor variant peptides were not recognized by specific T-cells when pulsed exogenously on donor EBV-LCL, suggesting that immunogenicity of the patient antigen is attributed to induction of T-cells expressing TCR that are able to discriminate between polymorphic residues. For the remaining antigen, i.e., LB-PIP4K2A-1S, we demonstrate that immunogenicity is at least partially mediated by intracellular cleavage of the donor variant by a protease named asparagine endopeptidase (AEP), thereby precluding its surface presentation. Interestingly, AEP is expressed at low levels in primary acute myeloid leukemia (AML), which hinders differential cleavage of polymorphic peptides and leads to strong T-cell recognition of both patient- and donor-type antigens when presented by HLA class II.

MATERIALS AND METHODS

Hematopoietic Cell Isolation

Peripheral blood was obtained from healthy individuals and patients with leukemia after approval by the Leiden University Medical Center Institutional Review Board and informed consent according to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Isopaque separation and cryopreserved. CD34+ hematopoietic stem cells were isolated from stem cell grafts and CD33+ AML blasts from peripheral blood or bone marrow by flow cytometric cell sorting.

Cell Culture

MJS, SD1 (26), and EBV-transformed B-cell lines (EBV-LCL) were cultured in IMDM (Lonza BioWhittaker) supplemented with 10% fetal calf serum (FCS) (Cambrex), 1% penicillin/streptomycin (Lonza), and 1.5% L-glutamine (Lonza). T-cell clones were cultured in IMDM supplemented with 5% human AB serum (Anprotec), 5% FCS, and 100 IU/ml interleukin-2 (IL-2) (Chiron), and restimulated every 10–20 days with irradiated (50 gray) allogeneic PBMCs and 0.8 μg/ml phytohemagglutinin (PHA) (Oxoid).

Whole Genome Association Scanning

WGAS was performed as previously described (27). Briefly, a panel of 80 EBV-LCL was genotyped for 1.1 million SNPs using microarrays. T-cell recognition of the panel was measured after retroviral transduction with the HLA-DRB1*03:01 restriction molecule, and recognition patterns were compared with SNP genotype data. The level of matching between the patterns of T-cell recognition and SNP genotypes was calculated by Fisher's exact test using PLINK WGA analysis software (28). For WGAS, test results needed to be categorized in two distinct groups. Therefore, EBV-LCL were divided into positive and negative groups based on the level of IFN-γ production. WGAS was performed by combining T-cell recognition with SNP genotyping data.

Sequencing of PIP4K2A Genotype

Genomic DNA of patient- and donor-derived EBV-LCL, MJS, primary AML, and primary healthy $CD34^+$ hematopoietic stem cells was isolated by using QIAamp DNA Blood mini kit (Qiagen) mini columns. DNA concentrations have been measured using a NanoDrop Microvolume spectrophotometer (Thermo Fisher). PCR amplification (initial denaturation 2 min 30 s; 94°C; 40 cycles: denaturation 45 s; 95°C, annealing 45 s; 60°C, elongation 1 min 30 s; 72°C; final elongation 10 min, 72°C) was performed using 100 ng of genomic DNA with the following primers: 5′-GCC AAA GAA CTG CCA ACT CT-3′ (forward primer) and

5′-GGC CTC TCC ACT GAC TGT TC-3′ (reverse primer). PCR products were purified using the QIAquick PCR purification kit (Qiagen). Sanger sequencing was performed using 25 pmol of the forward and reverse primer, respectively, and 200 ng of purified PCR product. Sequence reaction was performed by Eurofins Genomics (Luxembourg).

Flow Cytometry

For isolation of retrovirally transduced cells carrying the marker gene CD2, APC-labeled anti-murine CD2 (clone RM2-5; Biolegend) was used. Healthy hematopoietic stem cells were isolated based on expression of CD34 using PE-labeled anti-CD34 (8G12; BD) and acute myeloid leukemic cells were isolated based on expression of CD33 using BV421-labeled anti-CD33 (WM53; BD). Cells were washed twice with phosphate-buffered saline containing 2% FCS and incubated with fluorochrome-conjugated monoclonal antibodies for 30 min at room temperature. Data acquisition was performed on a fluorescence-activated cell sorter Canto II and a fluorescence-activated cell sorter BD FACS Aria (BD Biosciences). Forward scatter/side scatter was used for gating on viable cells. FSC-H/-A was used for doublet exclusion. Data were analyzed with Kaluza 2.1. (Beckman Coulter).

Retroviral Constructs and Transduction

All experiments involving retroviral vectors were approved by the government and handled according to biosafety level 2. All constructs were cloned in MP71 retroviral vectors containing different marker genes. PIP4K2A was linked to the GFP marker gene, AEP to murine ΔCD2, and HLA-DRB1*03:01 to ΔNGFR. All constructs were verified by sequencing. Retroviral supernatant was obtained by transfecting wild-type *Φ*nx-A packaging cells as previously described (29), with the exception that the X-tremeGENE HP DNA Transfection Reagent transfection kit (Roche Diagnostics) was used. Non-tissue culture-treated culture plates were coated (overnight at 4°C) with RetroNectin (30 μg/ml) Recombinant Human Fibronectin Fragment (Takara) before harvested retroviral supernatants were applied and centrifuged at 32°C for 2 h at 2,000 *g*. After centrifugation, cells ($1–5 \times 10^5$) were directly transferred into the infectious supernatant and marker gene expression measured after 3 days by flow cytometry.

Antigen Presentation Assays

Stimulator cells (3×10^4 cells/well or as indicated) were co-incubated with the $CD4^+$ T-cell clone (5×10^3 cells/well or as indicated) overnight at 37°C in 96-well plates in duplicates. For peptide loading, stimulator cells were incubated with indicated peptide concentrations for 2 h at 37°C before T-cell clones were added. For shutdown of the tet-off system, cells were cultured in the presence of 50 μg/ml doxycycline and washed twice before co-incubation with the T-cell clone. Cytokine release was measured after overnight incubation in 100-μl supernatants by IFN-γ ELISA following the instructions of the manufacturer (Thermo Fisher Scientific).

Enzymatic Activity of AEP

To determine the activity of AEP, cells were lysed by three cycles of freezing and thawing. Subnuclear fractions were collected by harvesting supernatants after centrifugation at 10,000 *g* for 20 min. Protein concentrations were measured using the BCA protein assay (Thermo Scientific). Cellular lysates (2 and 5 μg) were resuspended in sodium citrate buffer (50 mM, pH 5.5; 5 mM DTT, 0.1% CHAPS). Z-Ala-Ala-Asn-AMC (10 μM; Bachem) was added to the lysates for 30 min at room temperature. Developing fluorescence (excitation 370 nm; emission 460 nm) was measured for 10 min on a NOVOstar analyzer (BMG labtech).

Microarray Gene Analysis

Total RNA was isolated using small- and micro-scale RNAqueous isolation kits (Ambion) and amplified using the TotalPrep RNA amplification kit (Ambion). After preparation using the whole-genome gene expression direct hybridization assay (Illumina), cRNA samples were dispensed onto Human HT-12 v3 Expression BeadChips (Illumina). Hybridization was performed in the Illumina hybridization oven for 17 h at 58°C. Microarray gene expression data were analyzed using R 2.15. Normalization was done in the lumi package, using the variance stabilizing transformation and quantile normalization (30).

Statistical Analysis

Data were analyzed with Prism 8.3.0 (GraphPad Software Inc.). If not otherwise stated, for statistical analysis, at least three individual experiments were performed and the unpaired *t*-test was applied. Statistical significance was indicated as $^*P < 0.05$ or $^{**}P < 0.01$. For WGAS, the level of matching between T-cell recognition pattern and SNP data was calculated according to Fisher's exact test.

RESULTS

Identification of Four New HLA Class II-Restricted Minor Histocompatibility Antigens by WGAS

The target antigens of four $CD4^+$ T-cell clones were identified by WGAS. All T-cell clones have been shown to be specific for minor histocompatibility antigens by recognizing patient but not donor EBV-LCL. Clone 100 has been isolated from bone marrow of patient 3,087, 5 weeks after donor lymphocyte infusion (DLI) for relapsed chronic myeloid leukemia (CML) after alloSCT (9) and was restricted to HLA-DRB1*03:01. Clone 8-10A and clone 8-15 were isolated from peripheral blood of patient 2,877, 4 weeks after DLI for relapsed CML after alloSCT and were both restricted to HLA-DQB1*06:02. Finally, clone 15-18, which was also HLA-DQB1*06:02-restricted, was isolated from patient 5,852 who was treated with DLI for mixed chimerism 6 months after alloSCT for myelodysplastic syndrome refractory anemia with excess of blasts type 2. To identify the target antigens of these T-cell clones, we tested reactivity against a panel SNP-genotyped EBV-LCL either transduced with HLA-DRB1*03:01 (clone 100; **Figure 1A**) or endogenously expressing HLA-DQB1*06:02 (clones 8-10A, 8-15, and 15-18) and correlated T-cell recognition data with SNP

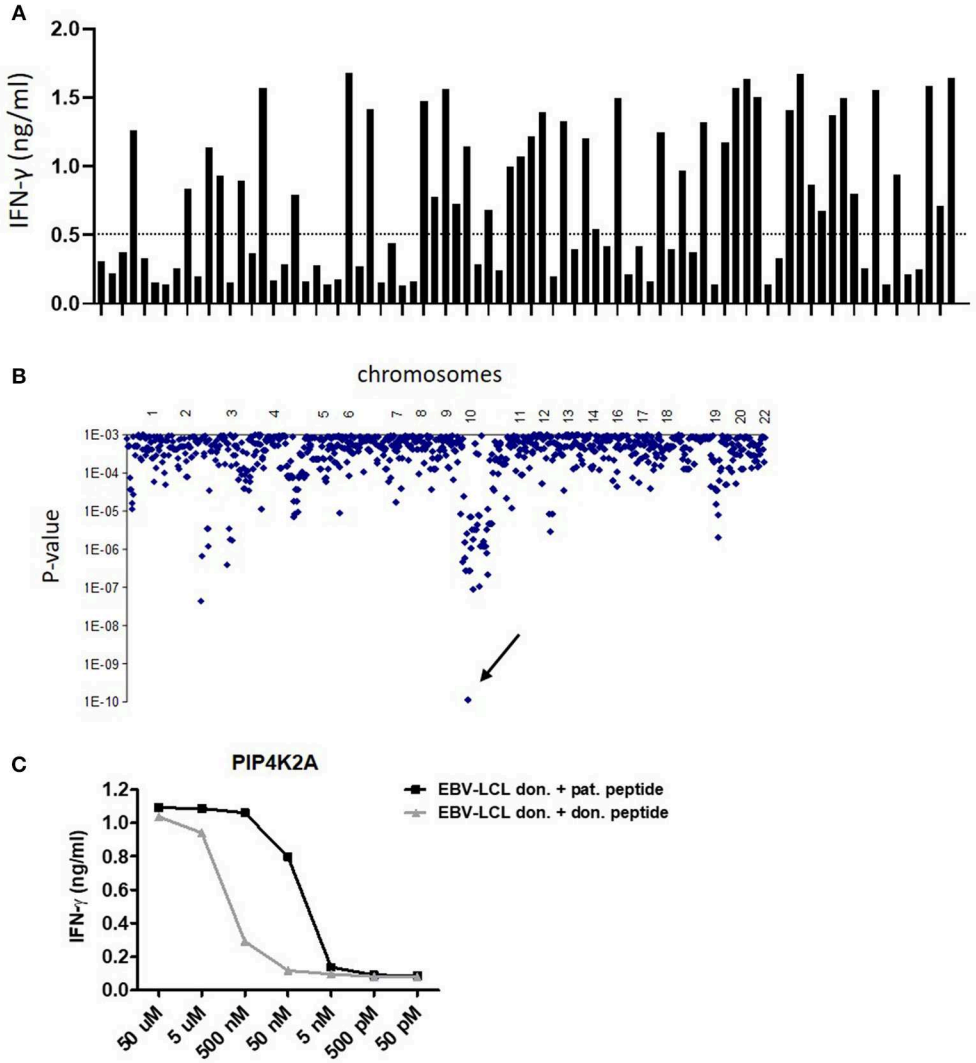

FIGURE 1 | Identification of LB-PIP4K2A-1S as new HLA class II-restricted minor histocompatibility antigen by whole genome association scanning. **(A)** T-cell recognition of a panel of 80 HLA-DRB1*0301 transduced EBV-LCL. Bars represent the level of IFN-γ (ng/ml) in ELISA released by clone 100 upon co-incubation with the different EBV-LCL. **(B)** Whole genome association scanning of the recognition data for 80 HLA-DRB1*0301 transduced EBV-LCL and the corresponding SNP data revealed one strongly correlating missense SNP in PIP4K2A (rs10828317) (arrow). The *y*-axis indicates *P*-values for the significance of association between SNPs and T-cell recognition of EBV-LCL. The *x*-axis represents the 1 million tested SNPs as distributed over the human chromosomes. **(C)** Synthetic peptides of patient and donor variants were loaded on donor EBV-LCL in indicated concentrations and recognition by T-cell clone 100 was measured in IFN-γ ELISA. The LB-PIP4K2A-1S-specific T-cell clone 100 also showed recognition of the donor PIP4K2A-1N variant at peptide concentrations over 50 nM.

genotypes of the respective EBV-LCL (27). The level of matching was calculated according to Fisher's exact test.

For clone 100, one SNP genotype significantly associated with T-cell recognition with a *P*-value $< 1 \times 10^{-9}$ (**Figure 1B**). This was a coding missense SNP (rs10828317) in PIP4K2A (HGNC ID: 8997). Sequencing cDNA of patient and donor EBV-LCL for the region encompassing the SNP confirmed heterozygosity in patient cells at this position (G/A), whereas donor cells were homozygous A/A. This A-to-G transition creates an Asn-to-Ser substitution at amino acid position 251 (N->S251) of the PIP4K2A protein.

For clone 8-10A, two SNPs (rs4242391 and rs1133782) significantly associated with T-cell recognition with *P*-values $<1 \times 10^{-9}$. One of these SNPs (rs1133782) is a missense variant in TNFRSF10D (HGNC ID: 11907). The T-to-C transition creates a Leu-to-Ser substitution at amino acid position 310 (L->S310) of the TNFRSF10D protein.

Two SNP genotypes (rs4740 and rs4905) with *P*-values $<1 \times 10^{-8}$ associated with T-cell recognition by clone 8-15. Rs4740 is a missense variant in EBI3 (HGNC ID: 3129) and the G-to-A transition translates into a Val-to-Ile substitution at amino acid position 201 (V->I201) of the EBI3 protein.

Also for clone 15-18, two SNP (rs17700475 and rs6441226) associated with T-cell recognition with *P*-values $< 1 \times 10^{-9}$. SNP rs6441226 is an intron variant in MFSD1 (HGNC ID: 25874), which has a minor allele frequency (MAF) of 0.1478 in 1,000 Genomes. Searching the SNP database for a missense SNP with a similar MAF revealed SNP rs28364680 that encodes a C-to-T transition leading to a Pro-to-Ser substitution.

T-cell recognition of donor EBV-LCL loaded with synthetic peptides surrounding the patient and donor variants confirmed T-cell specificity for the patient variants and validated these peptides as minor histocompatibility antigens (**Supplemental Table 1**). T-cell clones 8-10A, 8-15, and 15-18 failed to recognize donor peptide variants (**Supplemental Figure 1**). T-cell clone 100, however, showed strong recognition of the donor variant (INEGQKIYIDDN**N**KKVFLE) at peptide concentrations >50 nM, while there was no recognition at lower peptide concentrations (**Figure 1C**). The patient peptide variant (INEGQKIYIDDN**S**KKVFLE) was recognized at peptide concentrations >5 nM. These data indicate that the TCR of clone 100 is less able to discriminate between polymorphic peptides than the TCR of clones 8-10A, 8-15, and 15-18, suggesting that also other mechanisms are involved in differential recognition of patient and donor EBV-LCL.

Surface Presentation of Donor Variant PIP4K2A-1N Is Hampered by AEP-Mediated Cleavage

To investigate whether donor EBV-LCL fail to present the donor variant peptide as a consequence of low gene expression, we established overexpression of PIP4K2A by retroviral transduction. Interestingly, overexpression of full-length PIP4K2A encoding donor variant PIP4K2A-1N induced only low recognition of donor EBV-LCL, while significant recognition was observed for transduced melanoma Mel-Juso (MJS) cells (**Figure 2**). This suggests that another intracellular mechanism than gene expression is involved in differential recognition of LB-PIP4K2A-1S and its allelic variant. As the donor PIP4K2A-1N variant is defined by an asparagine in substitution of a serine in the patient variant, we hypothesized that the donor peptide may be intracellularly cleaved by AEP (alias LGMN) as described for other antigens (26). We therefore expressed the full-length patient and donor genes for PIP4K2A in melanoma cell line SD1, which expressed a tet-off system with AEP under control of doxycycline (26). Testing T-cell recognition of these transduced SD1 cell lines revealed increased recognition of PIP4K2A-1N after treatment with doxycycline, which shuts down AEP enzyme activity (**Figure 3A**). To confirm these data, we cloned the AEP gene and retrovirally overexpressed the protein in MJS cells co-transduced with the PIP4K2A variants. T-cell recognition of PIP4K2A-1N was significantly decreased upon overexpression of AEP, while recognition of LB-PIP4K2A-1S was not affected (**Figure 3B**). These data indicated that donor variant PIP4K2A-1N is enzymatically cleaved by AEP, thereby hampering presentation and T-cell recognition of the epitope at the cell surface.

Low Expression of AEP in Primary AML and Healthy CD34$^+$ Hematopoietic Stem Cells

We hypothesized that enzymatic cleavage of donor variant PIP4K2A-1N could potentially be overcome by high expression of the substrate, i.e., PIP4K2A, or low expression or activity of AEP, and therefore analyzed mRNA expression of PIP4K2A and AEP by microarray gene analysis (**Figure 4** and **Supplemental Table 2**). In addition to MJS and various other cancer cell lines, we analyzed gene expression in a wide variety of primary cell types of hematopoietic and non-hematopoietic origin, including bone marrow and PBMC, primary T- and B-cells, monocytes, macrophages, monocyte-derived immature and mature dendritic cells, healthy CD34$^+$ hematopoietic stem cells, acute and chronic myeloid leukemia, acute and chronic lymphocytic leukemia, and multiple myeloma as well as

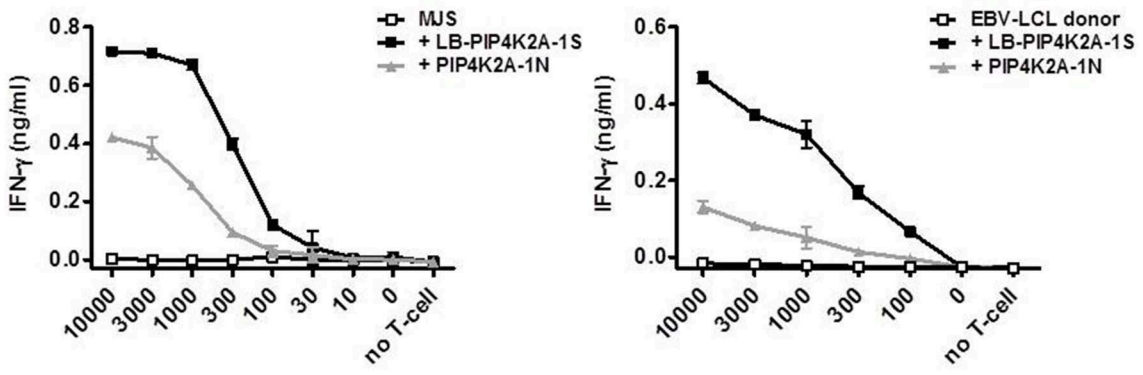

FIGURE 2 | Cell type-specific recognition of endogenously expressed PIP4K2A-1N donor variant. Full-length genes encoding LB-PIP4K2A-1S and PIP4K2A-1N were retrovirally transduced in MJS **(left)** or donor EBV-LCL **(right)** and transduced cells were isolated by flow cytometric sorting based on expression of marker gene GFP. T-cell recognition by clone 100 of the different cell lines was measured in IFN-γ ELISA. Indicated is the release of IFN-γ (ng/ml) by T-cell clone 100 upon co-incubation with indicated numbers of transduced MJS cells or EBV-LCL. Indicated are mean and standard deviation of duplicate wells. One representative of three independent experiments is shown.

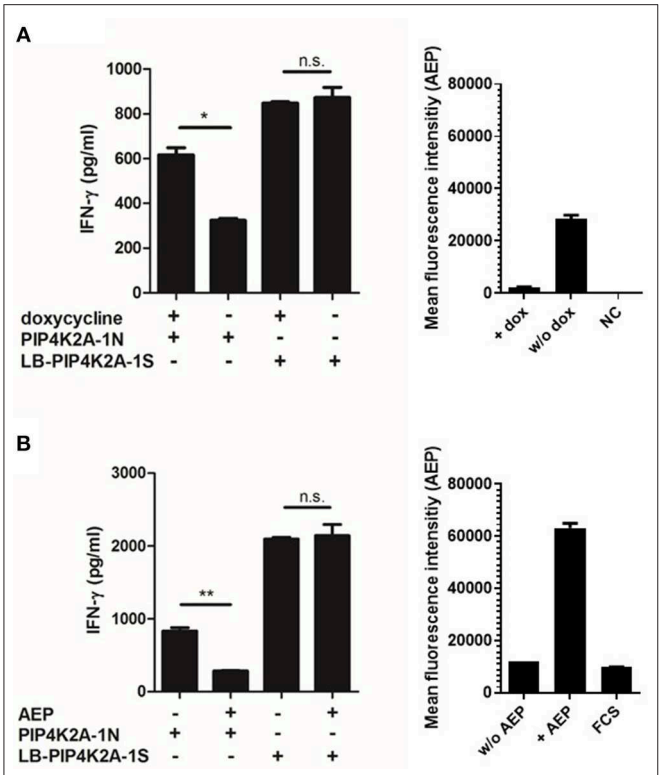

FIGURE 3 | Recognition of endogenously expressed PIP4K2A-1N donor variant correlates with activity of asparagine endopeptidase AEP. **(A)** SD1 cells expressing a tet-off vector system with AEP were retrovirally transduced with full-length genes encoding the LB-PIP4K2A-1S patient variant or PIP4K2A-1N donor variant and tested for T-cell recognition with and without prior doxycyclin (dox) treatment (left). Enzyme activity with and without doxycyclin treatment has been measured in cellular lysates (right). NC, negative control. **(B)** T-cell recognition of MJS retrovirally transduced with full-length genes encoding LB-PIP4K2A-1S patient variant or PIP4K2A-1N donor variant with or without additional transduction of AEP (LGMN). T-cell recognition has been tested in IFN-γ ELISA (left). Enzyme activity of AEP has been measured in transduced cell lines and fetal calf serum (FCS) as negative control (right). $^{*}P < 0.05$; $^{**}P < 0.01$ (unpaired *t*-test). One representative of three independent experiments is shown.

primary fibroblasts, keratinocytes, proximal tubular epithelial cells, human umbilical vein endothelial cells, melanocytes, hepatocytes, and gut, lung, bile duct, and cornea epithelial cells. Various non-hematopoietic cell types were also cultured in the absence or presence of IFN-γ to mimic an inflammatory environment. Interestingly, we observed low expression of AEP especially in primary AML and $CD34^+$ hematopoietic stem cells, while PIP4K2A expression was less variable.

Primary AML Elicit T-Cell Reactivity Independent of Their SNP Status

Since AEP expression is low in primary AML and $CD34^+$ hematopoietic progenitor cells, we tested whether T-cell clone 100 could recognize PIP4K2A peptides on these cell types independent of the patient SNP. Primary AML cells were sorted on CD33 by flow cytometry and tested for recognition by T-cell clone 100 in IFN-γ ELISA. Data showed that all AML that were positive for the relevant restriction molecule HLA-DRB1*0301 were recognized by the T-cell clone independent of their SNP status (**Figure 5A**). In contrast, AML cells that lacked HLA-DRB1*0301 were not recognized. We also tested activity of AEP in these cells and confirmed low or absent enzyme activity, which is in line with low mRNA expression in these cells. In addition to AML cells, T-cell recognition of primary $CD34^+$ hematopoietic progenitors was tested. In contrast to AML, donor variant PIP4K2A-1N was not recognized on $CD34^+$ cells, which is probably due to low HLA class II expression or other accessory molecules in these cells since exogenous peptide loading also induced only marginal T-cell recognition of LB-PIP4K2A-1S. In contrast, a CMV derived antigen peptide presented in HLA-A*02:01 mediated strong T-cell recognition (**Figure 5B**). In conclusion, the data showed that T-cell clone 100 recognizes primary AML independent of their SNP status, thereby confirming surface presentation of donor variant PIP4K2A-1N in the absence of AEP.

DISCUSSION

T-cells directed against minor histocompatibility antigens are well established to mediate strong immune responses both against residual malignant cells and potentially against healthy non-malignant cells of the patient. A further understanding of these T-cell responses is crucial for eventually separating the effects of GvL and Graft vs. host disease. We here present four new HLA class II-restricted minor histocompatibility antigens identified by WGAS. Furthermore, we demonstrate that one of the new antigens is differentially presented on patient and donor cells due to intracellular cleavage of the donor peptide by AEP. This enzyme is not or less active in AML due to low expression of the endopeptidase. As a result, AML cells can be targeted by donor T-cells independent of the SNP status. These data illustrate that endopeptidases can play a role in cell type-specific intracellular processing and presentation of HLA class II antigens and provide evidence that after alloSCT, donor T-cells for minor histocompatibility antigens can also target the respective allelic variants as cell type-specific autoantigens.

Although it has been shown for various HLA class I-restricted minor histocompatibility antigens that their immunogenicity is based on differential intracellular processing of patient and donor variant peptides (21, 22, 24), all so far identified HLA class II-restricted minor histocompatibility antigens are recognized by T-cell receptors that fail to react with donor variants. Also, for three new HLA class II antigens identified here, donor variants are not recognized when loaded as exogenous peptides, indicating that patient and donor peptides are differentially recognized by the T-cell receptor as expressed by the specific T-cell clone. Alternatively, it is also possible that the donor peptide does not sufficiently bind to the HLA molecule and is therefore not presented, as described for HA-1 (25). However, for one new HLA class II antigen, i.e., LB-PIP4K2B-1S, we observed that its donor variant PIP4K2A-1N is strongly recognized when pulsed as exogenous peptide, suggesting involvement of an intracellular processing mechanism in differential recognition

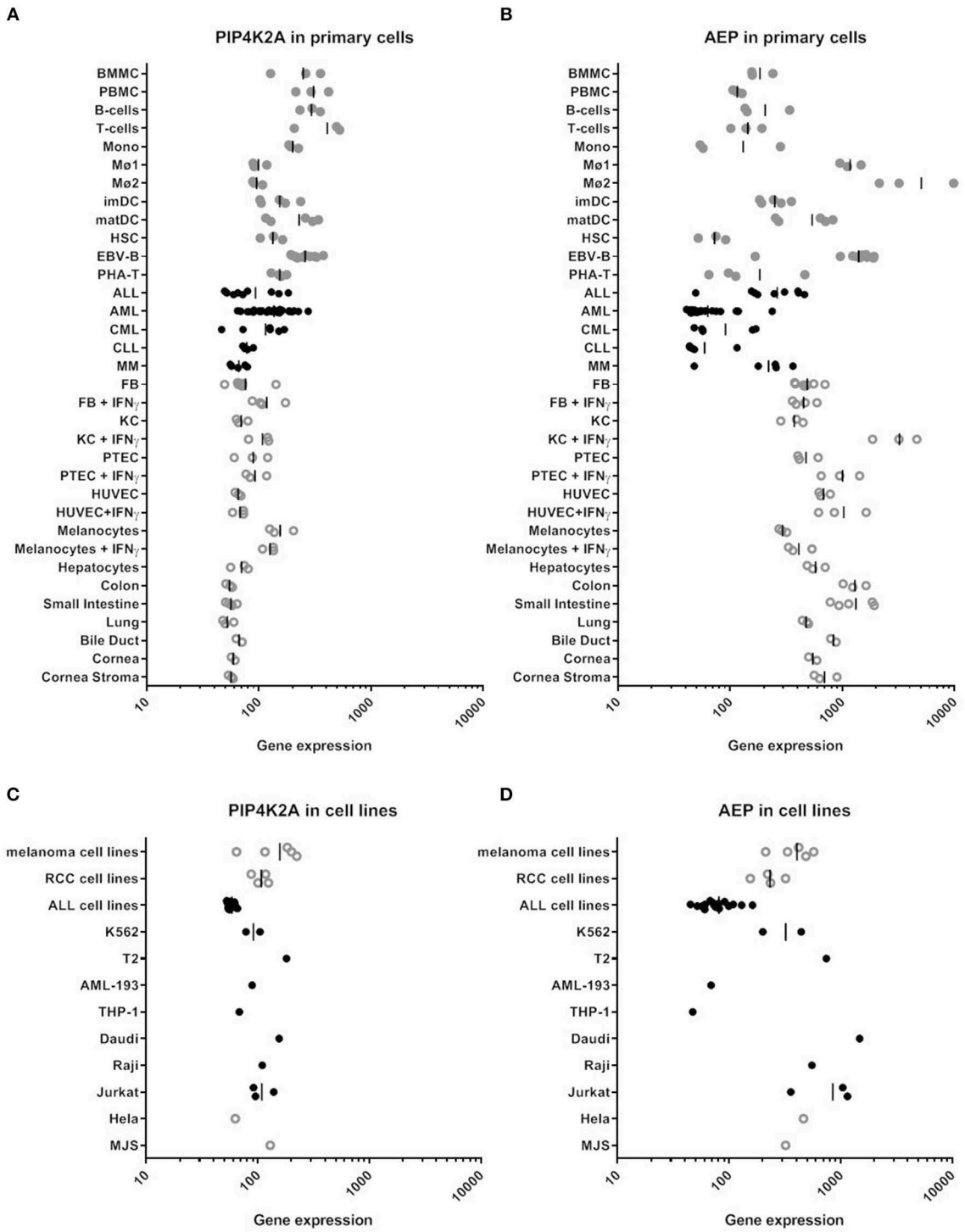

FIGURE 4 | RNA expression of PIP4K2A and AEP in various human cell types by microarray gene analysis. **(A)** PIP4K2A gene expression in primary cells. **(B)** AEP gene expression in primary cells. **(C)** PIP4K2A gene expression in cell lines. **(D)** AEP gene expression in cell lines. Healthy and malignant hematopoietic cells are represented by gray and black dots, respectively, while open symbols represent non-hematopoietic cell types. Gene expression was measured on Illumina HT-12 BeadChips as described previously (30). BMMC, bone marrow mononuclear cells; PBMC, peripheral blood mononuclear cells; Mono, monocytes, Mø1, type 1 macrophages; Mø2, type 2 macrophages; imDC, immature dendritic cells; matDC, mature dendritic cells; HSC, hematopoietic $CD34^+$ stem cells; EBV-B, EBV-transformed B cells; PHA-T, PHA-stimulated T-cells; ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; MM, multiple myeloma; FB, fibroblasts; KC, keratinocytes; PTEC, proximal tubular epithelial cells; HUVEC, human umbilical vein endothelial cells.

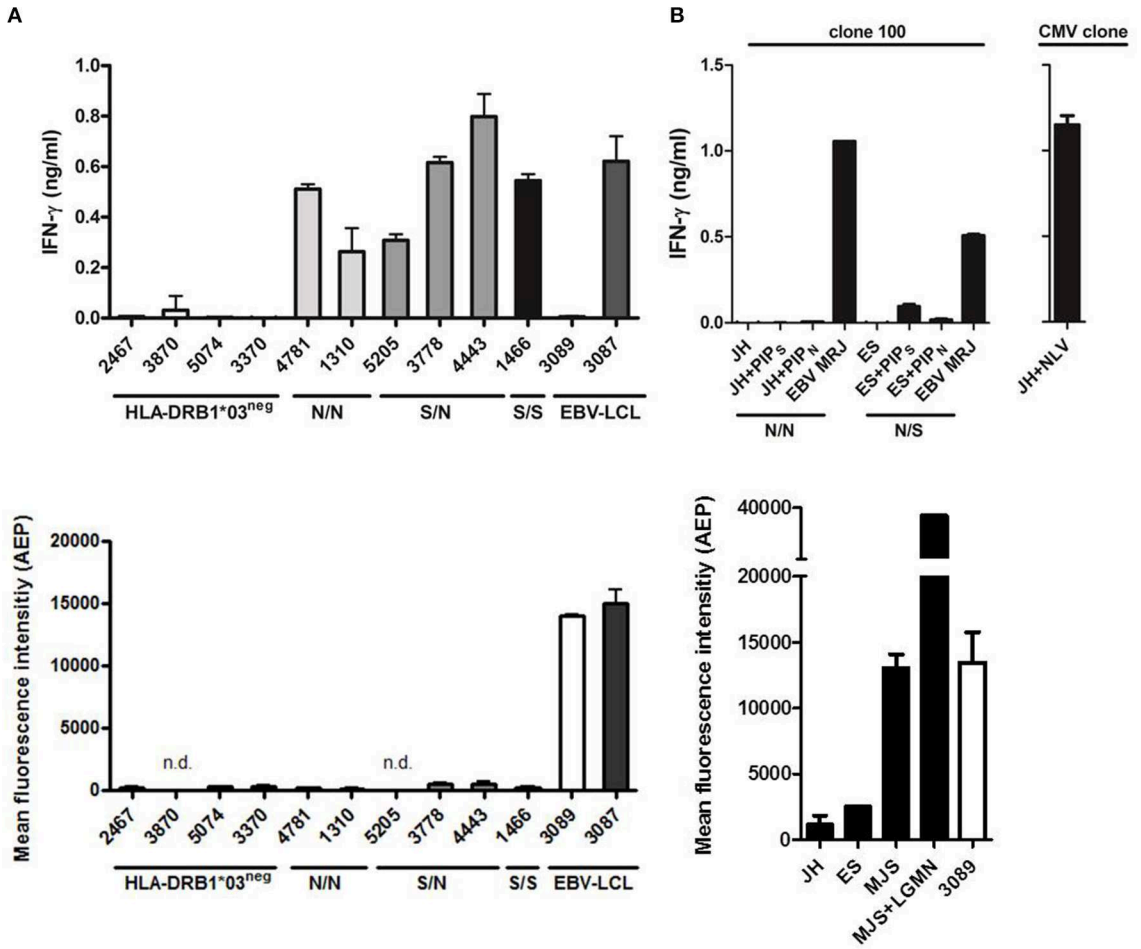

FIGURE 5 | Primary human AML are recognized independent of the SNP for PIP4K2A. **(A)** Recognition of primary human AML by T-cell clone 100 as tested in IFN-γ ELISA (top panel). PIP4K2A genotypes [N/N (light gray), S/N (dark gray), or S/S (black)] are depicted for HLA-DRB1*03 positive primary AML. EBV-LCL 3087 is derived from the patient from whom T-cell clone 100 has been isolated, and EBV-LCL 3089 is from its stem cell donor. Enzyme activity of AEP has been measured in corresponding AML cell lysates (bottom). n.d., not determined. **(B)** T-cell recognition of primary human $CD34^+$ cells has been tested by IFN-γ ELISA (top panel). PIP4K2A genotypes (N/N, S/N, or S/S) are depicted. Both patients are typed positive HLA-DRB1*03:01, patient JH is in addition typed positive for HLA-A*02:01 and viability of $CD34^+$ cells is confirmed by recognition of CMV peptide NLV in HLA-A*02:01. Enzyme activity of AEP in CD34 cell lysates is depicted (bottom). One representative of two independent experiments is shown.

of patient and donor variant. Of note, peptide titration still showed a marked difference in T-cell activation between LB-PIP4K2A-1S and its donor variant, indicating that intracellular processing is not the only mechanism and that the affinity of the T-cell receptor as expressed by the specific T-cell clone also contributes to differential recognition of patient and donor peptides. However, the observation that AEP strongly affects endogenous presentation of donor variant PIP4K2A-1N, but not of LB-PIP4K2A-1S, confirmed involvement of this endopeptidase in intracellular processing of the HLA class II antigen.

A role for AEP in antigen processing has already been described for other antigens. For tetanus toxin C fragment (TTCF), it has been demonstrated that deamidation of an asparagine residue hinders enzymatic cleavage by AEP, thereby perturbing antigen presentation (31). Whereas cleavage by AEP enhances antigen presentation for TTCF, presentation of LB-PIP4K2A-1S is disrupted. Similarly, it has been shown for myelin basic protein (MBP), which is an autoantigen in the inflammatory demyelinating disease multiple sclerosis, that autoreactive T-cells can evade central tolerance due to enzymatic cleavage of the autoantigen in the thymus (26). It has been suggested that under certain circumstances, these MBP-specific T-cells may become activated and induce autoimmunity. One of these circumstances may be the posttranslational modification of the asparagine residue by deamidation, thereby hindering cleavage and enhancing MBP antigen presentation. Non-enzymatic deamidation of asparagine to aspartic acid is the most commonly observed posttranslational modification in proteins. Although the C-terminally flanking lysine in PIP4K2A-1N is not ideal, deamidation of the asparagine can occur at intermediate turnover

rates (32) and may therefore contribute to surface presentation of the donor variant.

Apparently, in the stem cell donor for patient 3,087, a T-cell expressing a TCR for donor variant PIP4K2A-1N has evaded central tolerance, suggesting that the donor variant is not expressed during thymic selection. This is in line with our observation that even non-physiological levels of overexpression of the donor variant does not lead to strong T-cell activation. This, together with the observation that the stem cell donor and the transplanted patient lack any signs of autoimmunity, indicates that donor variant PIP4K2A-1N is not or not sufficiently presented on all or the majority of healthy tissues *in vivo*.

Thinking about therapeutic approaches, it is tempting to speculate that the TCR as expressed by the T-cell clone for LB-PIP4K2A-1S could be used for gene therapy to treat AML in patients who are homozygous for the donor variant (N/N) without need of prior transplantation. In this setting, the TCR is expected to selectively recognize PIP4K2A-1N as tumor antigen on low AEP-expressing leukemic cells, whereas all or the majority of healthy tissues are not expected to present the epitope due to intracellular AEP-mediated cleavage. While low expression of AEP in healthy $CD34^+$ hematopoietic stem cells is of concern, our *in vitro* experiments failed to show any T-cell recognition of these cells, probably due to a low overall HLA class II expression or lack of other accessory molecules stimulatory capacity in these cells. Moreover, we isolated the T-cell clone for LB-PIP4K2A-1S during GvL reactivity from a patient who was transplanted with $CD34^+$ hematopoietic stem cells from a PIP4K2A-1N homozygous donor, but had no signs of myeloablation. However, it cannot entirely be excluded that side effects may occur due to presentation of PIP4K2A-1N on certain cell types or healthy tissues as a result of low AEP expression or posttranslational modification of the asparagine residue. Although it is tempting to speculate, we would like to emphasize that we do not present PIP4K2A-1N as ideal target for immunotherapy of AML, since that conclusion requires additional *in vitro* and *in vivo* experiments, but rather as proof of concept that endopeptidases play a similar role in cell type-specific intracellular processing of HLA class II-restricted minor histocompatibility antigens as intracellular processing is known to be involved in presentation of HLA class I-restricted minor histocompatibility antigens (21–23).

Another question is why AML cells express low amounts of AEP and whether this can be influenced. Intriguingly, it was recently described that AEP is downregulated by PD-1 signaling in regulatory T-cells (33). It is tempting to speculate that this may also occur in tumor cells during immune evasion (34). This would make PIP4K2A-1N more attractive as a leukemia-associated antigen to be targeted by immunotherapy perhaps after or during treatment with cisplatin, a chemotherapeutic agent that is known to induce deamidation (35). Altogether, convincing evidence that PIP4K2A-1N can be used as a leukemia-specific target is lacking and demands further exploration using *in vivo* models. Nevertheless, discovery of LB-PIP4K2A-1S as a new minor histocompatibility antigen revealed an interesting role of an endopeptidase in cell type-specific intracellular processing of this HLA class II-restricted antigen and its donor variant, which may be explored in future immunotherapy of AML.

DATA AVAILABILITY STATEMENT

The analyzed datasets for this study are deposited in the NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE76340 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76340).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Leiden University Medical Center Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ANK performed and designed research and wrote the manuscript. JB, EL, CR, CB, SK, DM, EM, and MH performed research. AEK, CW, AM, JF, and MG designed research and wrote/reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00381/full#supplementary-material

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ORIGINAL RESEARCH
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Optimized Whole Genome Association Scanning for Discovery of HLA Class I-Restricted Minor Histocompatibility Antigens

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Patients undergoing allogeneic stem cell transplantation as treatment for hematological diseases face the risk of Graft-versus-Host Disease as well as relapse. Graft-versus-Host Disease and the favorable Graft-versus-Leukemia effect are mediated by donor T cells recognizing polymorphic peptides, which are presented on the cell surface by HLA molecules and result from single nucleotide polymorphism alleles that are disparate between patient and donor. Identification of polymorphic HLA-binding peptides, designated minor histocompatibility antigens, has been a laborious procedure, and the number and scope for broad clinical use of these antigens therefore remain limited. Here, we present an optimized whole genome association approach for discovery of HLA class I minor histocompatibility antigens. T cell clones isolated from patients who responded to donor lymphocyte infusions after HLA-matched allogeneic stem cell transplantation were tested against a panel of 191 EBV-transformed B cells, which have been sequenced by the 1000 Genomes Project and selected for expression of seven common HLA class I alleles (HLA-A*01:01, A*02:01, A*03:01, B*07:02, B*08:01, C*07:01, and C*07:02). By including all polymorphisms with minor allele frequencies above 0.01, we demonstrated that the new approach allows direct discovery of minor histocompatibility antigens as exemplified by seven new antigens in eight different HLA class I alleles including one antigen in HLA-A*24:02 and HLA-A*23:01, for which the method has not been originally designed. Our new whole genome association strategy is expected to rapidly augment the repertoire of HLA class I-restricted minor histocompatibility antigens that will become available for donor selection and clinical use to predict, follow or manipulate Graft-versus-Leukemia effect and Graft-versus-Host Disease after allogeneic stem cell transplantation.

Keywords: minor histocompatibility antigens, whole genome association scanning, allogeneic stem cell transplantation, HLA class I, graft versus host disease, Graft-versus-Leukemia effect, hematological diseases

INTRODUCTION

Allogeneic stem cell transplantation (alloSCT) has a curative potential for treatment of hematological malignancies (1, 2). After alloSCT, however, patients still face the risk of disease relapse as well as Graft-versus-Host Disease (GvHD), both contributing to morbidity and mortality. A strategy to reduce GvHD is to deplete donor T cells from the stem cell graft followed by delayed administration of donor lymphocyte infusions (DLI) after alloSCT in order to mitigate relapse (3). Donor T cells that are present in the stem cell graft or DLI induce beneficial Graft-versus-Leukemia (GvL) reactivity as well as undesired GvHD by targeting polymorphic peptides, designated minor histocompatibility antigens (4–6).

Minor histocompatibility antigens are peptides produced by single nucleotide polymorphisms (SNPs), which differ between patient and donor, and are presented by HLA molecules on the cell surface (4–6). They are similar to neoantigens with respect to amino acid changes that are recognized by the immune system, but are encoded by germline polymorphisms instead of somatic mutations (7). This has the advantage that minor histocompatibility antigens are shared between patients independent of the disease. However, in contrast to somatic mutations, which are restricted to tumor cells or only a subclonal population, polymorphisms are present in all tissues. Therefore, the tissue distribution of minor histocompatibility antigens is a relevant factor for the type of clinical response that is induced after alloSCT. Donor T cells recognizing antigens that are broadly expressed on malignant cells and healthy tissues may induce GvL reactivity as well as GvHD, while donor T cells targeting antigens that are only expressed on (malignant) hematopoietic cells selectively mediate a GvL effect without GvHD.

Identification of minor histocompatibility antigens in GvL and GvHD is important to enable strategies to separate the two clinical effects (8). Characterizing antigens that induce GvHD may enable selection of a donor that is matched with the patient for the encoding SNPs. Moreover, in case a patient is transplanted with a donor mismatched for these SNPs, T cells for ubiquitous antigens may be selectively depleted from the stem cell graft or DLI. Hematopoietic-restricted minor histocompatibility antigens are relevant for therapeutic approaches aimed at enhancing immunity against the malignancy, either by vaccination (e.g., in the clinical trials 2012-002435-28, 2018-002752-33, NCT02528682) or adoptive T cell therapy (e.g., NCT03091933 and NCT03326921). Discovery of the dominant repertoire of minor histocompatibility antigens in GvL and GvHD that are often mismatched between patients and donors is therefore highly relevant for optimal development of strategies to separate GvL and GvHD after alloSCT.

Currently, a total of 63 autosomal HLA class I minor histocompatibility antigens have been identified and confirmed as targets for T cell clones after alloSCT (5, 9–13). The first antigens were found by laborious methods, i.e., peptide elution (14), cDNA library screenings (15) and genetic linkage analyses (16). A significant improvement was achieved by introduction of whole genome association scanning (WGAs; **Figure 1**). Kamei et al. (17) tested T cell clones against a panel of Epstein-Barr virus-transformed lymphoblastoid cell lines (EBV-LCLs) and determined antigens by genetic mapping using the International HapMap Project. We refined the WGAs method by screening 1.1 million SNPs for association with recognition patterns of T cell clones against a panel of 80 EBV-LCLs, aimed at characterizing minor histocompatibility antigens presented by HLA-A*02:01 and B*07:02 (18). The SNPs for these EBV-LCLs were measured using a SNP-array and either directly encoded the antigen or served as markers in linkage disequilibrium with the antigen-encoding SNP that was not measured by the array. Using this approach, we discovered around 50% of the currently known HLA class I minor histocompatibility antigens (10–19).

In 2014, Oostvogels et al. (20) successfully identified an HLA class II minor histocompatibility antigen by scanning the genomes of 43 EBV-LCLs that were transduced with HLA-DPB1*04:01 and sequenced as part of the 1000 Genomes Project. Here, we used EBV-LCLs that were sequenced as part of the 1000 Genomes Project to optimize the WGAs approach for the discovery of HLA class I minor histocompatibility antigens. A total of 191 EBV-LCLs were selected, allowing the screening of around 11 million SNPs and small indels (MAF $>$ 0.01) in seven common HLA class I alleles (A*01:01, A*02:01, A*03:01, B*07:02, B*08:01, C*07:01, and C*07:02). We explored the potential of the panel to identify antigens with different allele frequencies in each of the seven HLA class I alleles and, as proof of principle, tested the new panel using T cell clones recognizing known minor histocompatibility antigens. Furthermore, the panel was tested using T cell clones for unknown antigens. A total of seven new minor histocompatibility antigens were successfully identified in eight different HLA class I alleles. These peptides include one antigen in HLA-A*23:01 and A*24:02, for which the panel was not specifically designed, and one antigen, for which identification failed by our previous method by scanning 1.1 million SNPs and small indels, thereby confirming the improved efficiency of the WGAs approach to discover HLA class I minor histocompatibility antigens.

MATERIALS AND METHODS

All experiments have been performed according to standard biosecurity and institutional safety procedures.

Patients

Peripheral blood and bone marrow samples were collected from six patients and their donors after approval by the LUMC Institutional Review Board (nos. P03.114, P03.173, and P04.003) and obtaining written informed consent according to the Declaration of Helsinki. The six patients underwent allogeneic stem cell transplantation for the treatment of acute myeloid leukemia (AML), chronic myeloid leukemia (CML) or myelodysplastic syndrome (MDS) and developed a clinical immune response after DLI characterized by GvHD or disappearance of patient cells in bone marrow or peripheral blood. Mononuclear cells were obtained by Ficoll-Isopaque gradient centrifugation and cryopreserved.

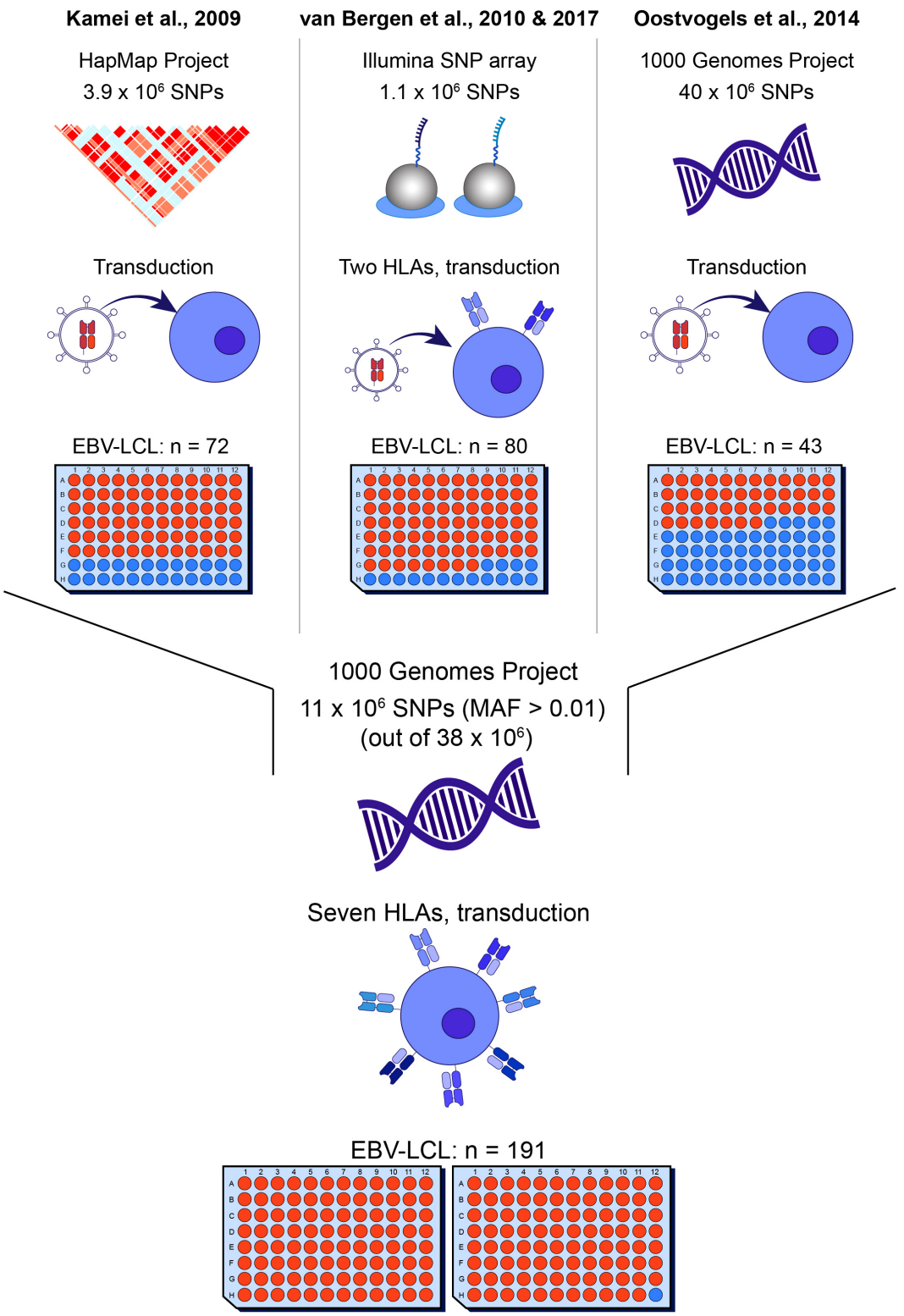


FIGURE 1 | Overview of whole genome association methods for identification of minor histocompatibility antigens. Whole genome association scanning has been utilized for discovering minor histocompatibility antigens based on screening of SNPs of the HapMap Project [Kamei et al. (17)], measured by Illumina SNP array [van Bergen et al. (18)] and the 1000 Genomes Project [Oostvogels et al. (20)], using 43–80 EBV-LCLs, which had to be retrovirally transduced with the respective HLA restriction molecule or expressed only two HLAs (HLA-A*02:01 and B*07:02). The optimized panel allows screening for all SNPs and small indels of the 1000 Genomes Project and covers seven common HLAs (HLA-A*01:01, A*02:01, A*03:01, B*07:02, B*08:01, C*07:01, C*07:02) without the need for viral transduction of the HLA molecule with a panel size of 191 EBV-LCLs.

T Cell Isolation and Culture

Peripheral blood mononuclear cells (PBMCs), which were obtained from patients at different time points after DLI during a detectable immune response, were thawed and enriched for T cells using an untouched pan-T cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec, Bergisch-Gladbach, Germany). Activated $CD8^+$ T cells were either sorted based on expression of HLA-DR as *in vivo* activation marker or CD137 as *in vitro* activation marker. For *in vitro* activation, the enriched T cells were first stimulated with irradiated (15 Gy) patient PBMCs (E:T ratio of 1:2), which had been taken prior alloSCT, for 2 days in T cell medium [TCM; IMDM (Lonza, Walksersville, MD, United States), 5% fetal bovine serum (FBS; Bodinco, Alkmaar, Netherlands), 5% pooled human serum (Sanquin, Amsterdam, Netherlands), 1.5% glutamine (200 mM; Lonza, Walksersville, MD, United States), 1% penicillin/streptomycin (P/S; 200 mM; Lonza, Verviers, Belgium), 0.5 μg/ml amphotericin B (Bristol-Myers Squibb, Munich, Germany), 2 ng/ml IL-7 (Miltenyi Biotec), 2 ng/ml IL-15 (Miltenyi Biotec) supplemented with 20 IU/ml IL-2 (Novartis, Arnhem, Netherlands)]. Activated T cells were then stained with FITC-conjugated CD8 (clone RPA-T8, BD/Pharmingen, Breda, Netherlands) and APC-conjugated CD137 (clone MOPC-21, BD/Pharmingen) or HLA-DR (clone G46-6, BD/Pharmingen) and sorted on a BD FACS Aria device. Subsequently, T cells were dispensed at concentrations of 1, 3 or 10 cells/well in 384-well plates (Greiner Bio-One, Alphen a/d Rijn, Netherlands) and re-stimulated for expansion with irradiated (50 Gy) allogeneic PBMCs as feeders (25,000 cells/well) and 0.8 μg/ml phytohemagglutinin (PHA; Remel Europe, Dartford, United Kingdom) in TCM supplemented with 120 IU/ml IL-2. Re-stimulation was repeated on day 7 with 50,000 feeders/well and PHA. Growing T cell clones were afterward expanded by re-stimulation every 2 weeks with feeders at a ratio of 1:3-5. Experiments were performed on day 10–14 after re-stimulation.

EBV-LCL Culture and Preparation

EBV-LCLs were generated from patient and donor PBMC or bone marrow samples using standard procedures and cultured in IMDM supplemented with 10% FBS, 1.5% glutamine and 1% P/S. A total of 191 EBV-LCLs from subjects sequenced as part of the 1000 Genomes Project were obtained from Coriell Cell Repositories, as part of the GEUVADIS project. In the GEUVADIS project, these EBV-LCLs have been analyzed by whole transcriptome RNA sequencing (21, 22). For the current project, cells were seeded in duplicate in 96-well plates at 60,000 cells/well. Plates were cryopreserved in multiple copies and used for WGAs as described below.

T Cell Reactivity Assays

T cell clones were tested for recognition of EBV-LCLs by co-incubating 2,000 T cells with 15,000 EBV-LCLs (E:T ratio 1:7.5) loaded with or without peptide in TCM without IL-7 and IL-15 supplemented with 20 IU/ml IL-2 overnight in 384-well plates. Recognition of target cells was determined by measuring IFN-γ in the supernatant by ELISA according to manufacturer's instructions (Sanquin, Amsterdam, Netherlands).

SNP Data Files for Whole Genome Association Scanning

Data files containing biallelic SNPs and small indels aligned to GRCh37 for the 191 EBV-LCLs were downloaded from https://www.ebi.ac.uk/arrayexpress/experiments/E-GEUV-1/files/genotypes/. For 184 of the EBV-LCLs, genotyping had been retrieved from 1000 Genomes phase 1, while the remaining 17 samples had been imputed from Omni 2.5M SNP array data (21). As described by Lappalainen et al. (21), Gencode V12 had been used to functionally reannotate all variants and QTL mapping had been done with linear regression, using genetic variants with >5% frequency in 1-megabase window and normalized quantifications transformed to standard normal. Permutations had been used to adjust the false discovery rate to 5%. Using the software Plink 1.90 (23), data files were converted to binary files, merged and filtered for 191 selected EBV-LCLs and SNPs with a minor allele frequency above 0.01, resulting in a total number of 10,955,109 SNPs. Whole genome association scanning analysis.

T-cell clones exclusively recognizing patient-derived, but not donor EBV-LCLs were selected and tested for reactivity against 191 EBV-LCLs of the 1000 Genomes Project. The 191 EBV-LCLs were selected for expression of seven common HLA class I alleles, including HLA-A*01:01, A*02:01, A*03:01, B*07:02, B*08:01, C*07:01, and C*07:02. HLA typing for individuals from the 1000 Genomes Project was obtained from Abi-Rached et al. (24). EBV-LCLs were also selected for co-expression of multiple of the seven HLA alleles to minimize the total number of cell lines required to compose a panel of EBV-LCLs aimed to include 50–100 cell lines per HLA. Since the panel is also used to determine HLA restriction of T cell clones, EBV-LCLs were as well specifically selected for expression of only one of two HLAs, which are often inherited in haplotype (e.g., B*07:02 and C*07:02; B*08:01 and C*07:01), in order to distinguish the molecules as separate HLA restriction alleles. The composition of the panel is shown in **Supplementary Table S1**. For each T-cell clone, EBV-LCLs were separated in positive and negative groups based on release of IFN-γ as measured by ELISA. HLA restriction of the T cell clones was determined by analyzing positive EBV-LCLs for shared expression of one of the HLA class I molecules as expressed by the patient and its donor. In a next step, positive and negative EBV-LCLs expressing the relevant HLA class I restriction allele were included in WGAs, while EBV-LCLs lacking the HLA allele were excluded. In WGAs, all 11 million SNPs were scanned for association with T cell recognition by means of the Fisher's exact test using Plink 1.90 taking around half a minute per run. The Fisher's exact calculates whether there is a significant difference in distribution of a SNP between EBV-LCLs that are recognized by the T cell versus EBV-LCLs that are not recognized by the T cell. Afterward, the reference SNP ID based on its chromosomal position and consequences of each SNP with a *p*-value cut-off at 10^{-5} were retrieved from Ensembl using biomaRt (25). SNPs

that strongly associated with T cell recognition were further investigated. Coding sequences surrounding SNPs of interest were extracted from ensembl.org, translated into six reading frames and corresponding peptide sequences were searched for predicted HLA-binders using NetMHC 4.0 (26). Visualization of data was done using in-house scripts in R.

Validation of New Minor Histocompatibility Antigens

Candidate peptides for minor histocompatibility antigens as well as their allelic variants were synthesized in house (purity >75%) and dissolved in DMSO. For validation, donor EBV-LCLs were pulsed with the peptides titrated in concentrations ranging from 50 μM to 1 pM and tested for recognition by the respective T cell clone by IFN-γ ELISA.

RESULTS

Design of Optimized WGAs to Identify Minor Histocompatibility Antigens

In order to develop a more efficient WGAs method to identify minor histocompatibility antigens, 191 EBV-LCLs of the 1000 Genomes Project were selected for expression of seven common HLA class I alleles. These alleles include HLA-A*01:01 (31.9%), A*02:01 (49.9%), A*03:01 (27.2%), B*07:02 (27.9%), B*08:01 (24.0%), C*07:01 (28.9%), and C*07:02 (30.8%) (percentages represent population frequencies calculated based on allele frequencies as reported in the Netherlands Leiden (n = 1305) population on www.allelefrequencies.net). In the finalized panel of 191 EBV-LCLs, each of the seven HLAs is expressed by 59–102 EBV-LCLs. In our selected panel of 191 EBV-LCLs, 176 cell lines are derived from individuals with Caucasian genetic background and 15 cell lines from individuals of Yoruba from Ibadan, Nigeria (**Supplementary Figure S1A**).

In order to evaluate the frequency of the selected HLAs in other human populations, we investigated HLA expression as reported for 2630 individuals in the five continental groups of the 1000 Genomes Project (24). In all human subpopulations, at least 43.5% of individuals express one or more of the seven selected HLAs (**Supplementary Figure S1B**), each expressed by at least 5% in each population with the exception of the East Asian group (**Supplementary Figure S1C**). In the European population, for which the panel has been designed, 87.2% of individuals express at least one of the selected HLAs and 56.9% of individuals express two or more common HLAs.

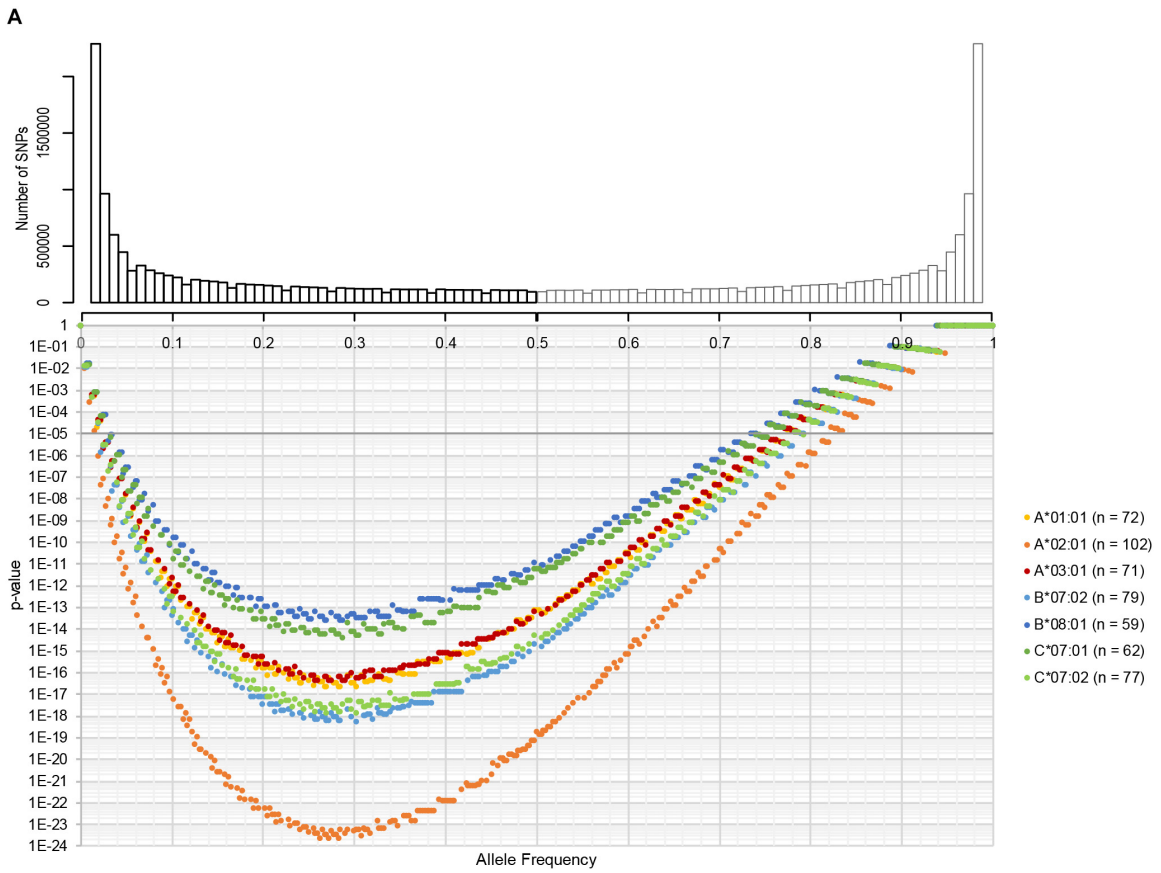
In silico Evaluation of Optimized WGAs to Identify Minor Histocompatibility Antigens

Using the 191 EBV-LCLs of the 1000 Genomes Project, we first performed an *in silico* analysis to evaluate the range of allele frequencies that can be identified for potential SNPs in seven common HLAs. For each HLA type, we predicted the number of EBV-LCLs that would be recognized (homo- or heterozygous for a specific SNP) or not (homozygous for its corresponding allelic variant). We then calculated the expected p-value for SNPs with different allele frequencies using the Fisher's exact test. Allele frequencies between 0.03–0.73 and 0.02–0.82 corresponding to the HLA expressed by the lowest (HLA-B*08:01, n = 59) and highest (HLA-A*02:01, n = 102) number of EBV-LCLs result in p-values below our selected arbitrary p-value cut-off at 10^{-5} (**Figure 2A**). These allele frequencies correspond to population frequencies of 0.06–0.92 and 0.04–0.96, respectively, indicating that for the seven selected HLAs, the new WGAs approach should allow identification of the vast majority of minor histocompatibility antigens that are frequently mismatched in patient-donor pairs.

In a next step, we evaluated whether the panel is adequate to identify the SNPs for 60 known SNP-encoded HLA class-I minor histocompatibility antigens (**Supplementary Table S2**). For each antigen-encoding SNP, we calculated the p-value based on genotyping of all EBV-LCLs in our panel expressing the relevant HLA class I restriction allele (**Figure 2B**). In congruence with our prediction, our panel allows identification of 38 out of 39 SNPs encoding known antigens that are presented by one of seven common HLAs with a p-value below 10^{-5}. Only one SNP that encodes HA-2 presented by HLA-A*02:01, which has a high population frequency of 0.99, reaches a p-value above 10^{-5}. In total, p-values below 10^{-5} were calculated for 40 antigens, indicating that two antigens could be identified that are presented by uncommon HLAs for which only 28–29 EBV-LCLs are included in the panel. These antigens are ACC-2D and ACC-1Y encoded by SNPs with allele frequencies of 0.24 and 0.25, resulting in p-values of 3.4×10^{-7} and 4.6×10^{-6} based on 28 (HLA-B*44:02) and 29 (HLA-A*24:02) EBV-LCLs in the panel, respectively. This implies that antigens presented by HLAs on a similar number of EBV-LCLs, namely, B*35:01 (n = 29), C*03:04 (n = 25), C*04:01 (n = 47), C*05:01 (n = 30) and C*06:02 (n = 27) can be identified, provided that the allele frequency of the SNP is between 0.17–0.44 (n = 25) and 0.05–0.68 (n = 47) corresponding to a population frequency of 0.32–0.68 and 0.10–0.89, respectively, in order to reach a p-value below 10^{-5}. The HLA class I alleles and corresponding range of allele frequencies for SNPs encoding minor histocompatibility antigens that can be directly identified by our optimized WGAs method are shown in **Supplementary Table S3**. ACC-2D is presented not only by HLA-B*44:02, but also by HLA-B*44:03, which increases the number of EBV-LCLs and lowers the p-value in the WGAs analysis to 8.2×10^{-10}. Except for HA-2, the remaining 19 antigens, for which p-values above 10^{-5} were obtained, are presented by infrequent HLA alleles for which only 2–29 EBV-LCLs were included in the panel. In conclusion, the *in silico* analysis supported that our optimized WGAs approach allows direct identification of the majority of minor histocompatibility antigens in seven common HLA class I alleles.

Performance of Optimized WGAs to Identify Known Minor Histocompatibility Antigens

We first assessed the performance of our new panel of 191 EBV-LCLs in WGAs by testing reactivity of five T cell clones



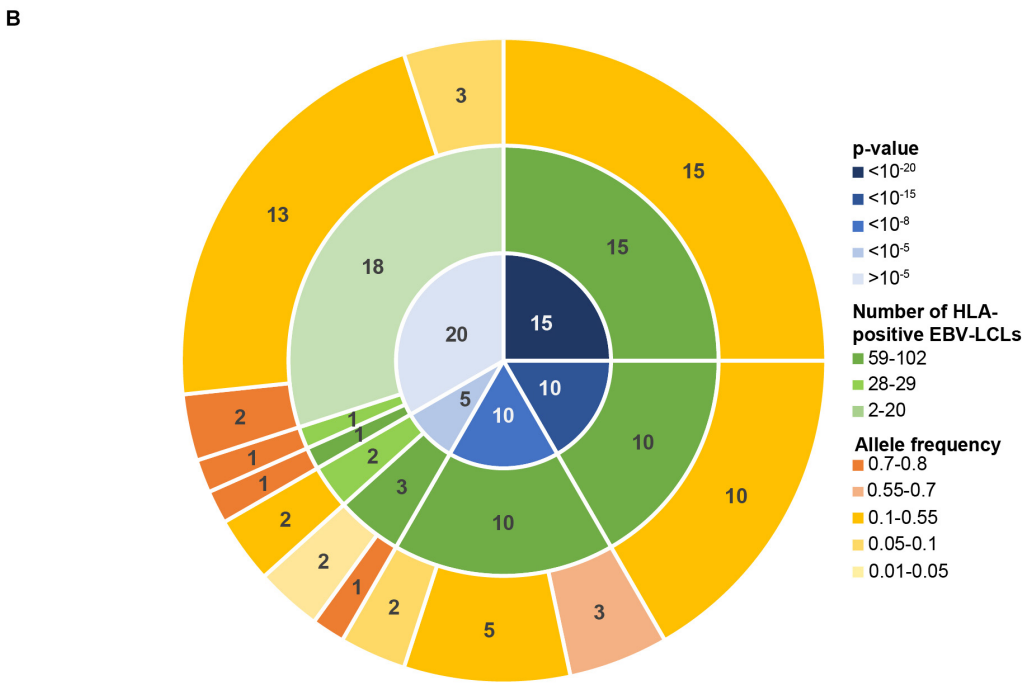


FIGURE 2 | Continued

FIGURE 2 | *In silico* evaluation of the optimized WGAs method to identify minor histocompatibility antigens. **(A)** In the optimized WGAs method, EBV-LCLs are scanned for 11 million SNPs with a minor allele frequency above 0.01. The upper graph shows the numbers of SNPs and their respective minor allele frequencies (black bars) in our panel of 191 EBV-LCLs from the 1000 Genomes Project. The major allele frequencies of the corresponding allelic variants (gray bars) are also shown. Based on the allele frequency and number of EBV-LCLs expressing HLA-A*01:01 (n = 72), A*02:01 (n = 102), A*03:01 (n = 71), B*07:01 (n = 79), B*08:01 (n = 59), C*07:01 (n = 62) and C*07:02 (n = 77), p-values were calculated using Fisher's exact test. For the seven common HLA class I restriction alleles, the sample size of the panel is sufficient to identify SNPs with allele frequencies ranging between 0.02–0.82 (HLA-A*02:01) and 0.03–0.75 (HLA-B*08:01) with a p-value below 10^{-5}. These allele frequencies correspond to population frequencies of 0.04–0.97 and 0.06–0.93, respectively, indicating that the majority of SNPs that are often mismatched in patient-donor pairs can be identified. **(B)** The panel of 191 EBV-LCLs from the 1000 Genomes Project was scanned for SNPs for 60 previously identified minor histocompatibility antigens that are presented by different HLA class I restriction alleles (**Supplementary Table S2**). Using Fisher's exact test, p-values below 10^{-5} were calculated for 38 of the 39 antigens that are presented by one of the seven HLA class I alleles for which 59–102 EBV-LCLs are included in the panel. The only antigen with a p-value above 10^{-5} was HA-2 in HLA-A*02:01, which has a high population frequency of 0.99. In total, p-values below 10^{-5} were calculated for 40 of the 60 antigens, indicating that the optimized WGAs approach also allows identification of 2 antigens for which only 26–28 EBV-LCLs are included. Of the 20 antigens with p-values above 10^{-5}, 19 antigens were presented by infrequent HLA alleles for which only 2–28 EBV-LCLs were included in the panel.

TABLE 1 | Detection of known minor histocompatibility antigens by the optimized WGAs approach.

Patient	Clone	Antigen	SNP	Gene	HLA	AF[1]	Peptide[2]	Illumina 1M array[3]	Predicted p-value	Observed p-value
9528	H.9B8	HA-3T	rs2061821	*AKAP13*	A*01:01	0.58	V[**T**/M]EPGTAQY	y	4.15E-12	4.15E-12
7103	93-23	HA-1	rs1801284	*HMHA1*	A*02:01	0.32	VL[**H**/R]DDLLEA	y	5.41E-25	5.41E-25
8031	p29-001	LB-NADK-1K	rs4751	*NADK*	A*03:01	0.37	AVHNGLGE[**K**/N]GSQA	n	2.40E-16	3.41E-15
5852	10-144-10	LB-ARHGDIB-1R	rs4703	*ARHGDIB*	B*07:02	0.46	LPRACW[**R**/P]EA	n	2.59E-15	1.36E-10
5596	76-116	LB-GEMIN4-1V	rs4968104	*GEMIN4*	B*08:01	0.21	FPALRFVE[**V**/E]	n	5.40E-14	1.42E-13

[1]*Allele frequency in panel.* [2]*Amino acid change that leads to antigen recognition is presented in brackets in bold.* [3]*Part of Illumina Human 1M-duo SNP array.*

for known minor histocompatibility antigens in different HLA class I alleles (**Table 1**). Of the five antigen-encoding SNPs, two had been directly identified as associating SNPs by our previous WGAs method in which 80 EBV-LCLs were scanned for 1.1 million SNPs. The remaining three antigen-encoding SNPs were indirectly identified via associating SNPs that are inherited in haplotype. Since the 80 EBV-LCLs were selected for co-expression of HLA-A*02:01 and B*07:02, the other three HLA class I alleles (A*01:01, A*03:01, and B*08:01) had to be retrovirally introduced. Here, we tested the five T cell clones for reactivity against the panel of 191 EBV-LCLs of the 1000 Genomes Project and measured release of IFN-γ by ELISA. EBV-LCLs expressing the relevant HLA restriction allele were divided into positive and negative groups based on T cell recognition. EBV-LCLs, which could not be assigned to either group due to intermediate IFN-γ signals as well as EBV-LCLs lacking expression of the relevant HLA restriction allele, were excluded from analysis. Using these T cell recognition patterns, all 11 million SNPs with a minor allele frequency above 0.01 were scanned for association using Plink 1.90. The SNPs that were identified with the new WGAs approach with p-values below 10^{-5} included all five antigen-encoding SNPs (**Supplementary Table S4**). Of the five SNPs, four were the strongest associating SNPs and one achieved second best p-value. Notably, comparing the genotypes of the antigen-encoding SNPs in EBV-LCLs with IFN-γ values measured by ELISA confirmed recognition patterns as expected for the T cell clones (**Figure 3**), and observed that statistical significance was only slightly less than predicted due to small numbers of excluded or incorrectly assigned EBV-LCLs (**Table 1**).

Identification of New Minor Histocompatibility Antigens by the Optimized WGAs Approach

Finally, the performance of the optimized WGAs method was evaluated for seven T cell clones recognizing unknown minor histocompatibility antigens. All T cell clones were isolated from patients with hematological malignancies who developed immune responses in the form of GvHD or disappearance of patient hematopoietic cells after treatment with T-cell depleted alloSCT and DLI. PBMCs after DLI were enriched for $CD3^+$ T cells and activated $CD8^+$ T cells were isolated either by CD137 2 days after *in vitro* stimulation with patient hematopoietic cells obtained prior to alloSCT or directly using HLA-DR as *in vivo* activation marker. Growing T cell clones were selected based on reactivity against patient-derived EBV-LCLs, but not donor EBV-LCLs either unpulsed or pulsed with a peptide mix for the 63 known HLA class I minor histocompatibility antigens as analyzed above. Seven patient-specific T cell clones were selected and tested for reactivity against the panel of 191 EBV-LCLs to identify the minor histocompatibility antigens. For each T cell clone, EBV-LCLs were divided into positive and negative groups based on IFN-γ secretion (**Supplementary Table S5**). To determine HLA restriction of the T cell clones, positive EBV-LCLs were analyzed for shared expression of one of the HLA molecules as expressed by patient and donor (**Figure 4**). Subsequently, WGAs was performed as described above to identify SNPs that strongly associated with T cell recognition (**Supplementary Table S6**). Strongly associating SNPs identified by our optimized WGAs method were further investigated for encoding peptides with predicted HLA-binding using NetMHC 4.0. Peptide candidates

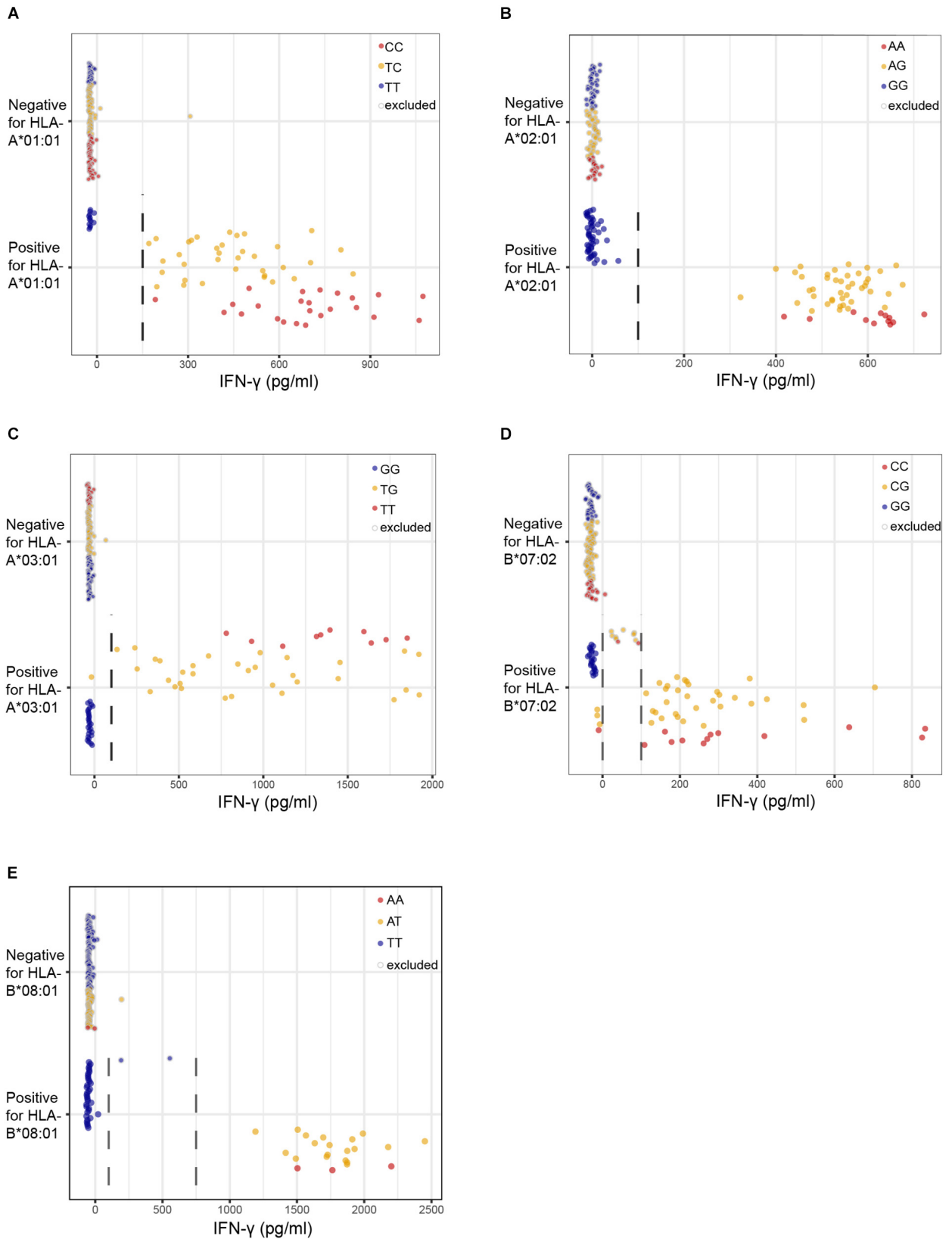


FIGURE 3 | Continued

FIGURE 3 | Performance of the optimized WGAs method to identify known minor histocompatibility antigens. T cell clones for HA-3T in HLA-A*01:01 **(A)**, HA-1 in A*02:01 **(B)**, LB-NADK-1K in A*03:01 **(C)**, LB-ARHGDIB-1R in B*07:02 **(D)** and LB-GEMIN4-1V in B*08:01 **(E)** were tested for reactivity against 191 EBV-LCLs from the 1000 Genomes Project by IFN-γ ELISA. EBV-LCLs are divided into groups based on presence or absence of the relevant HLA class I restriction allele. Dashed lines represent thresholds that were selected to divide the EBV-LCLs into negative or positive groups based on IFN-γ values for WGAs. EBV-LCLs with intermediate values, i.e., between two threshold lines, were excluded. EBV-LCLs that are homozygous or heterozygous for the antigen-encoding SNP are represented by red and orange dots, respectively. EBV-LCLs that are homozygous for the allelic variant are represented by blue dots. In WGAs, EBV-LCLs that are negative for the HLA restriction allele as well as EBV-LCLs with intermediate IFN-γ signals (gray border) were excluded from the analysis. The optimized WGAs approach correctly identified all antigen-encoding SNPs. The *p*-values as calculated by Fisher's exact test are shown in **Table 1** and **Supplementary Table S4**.

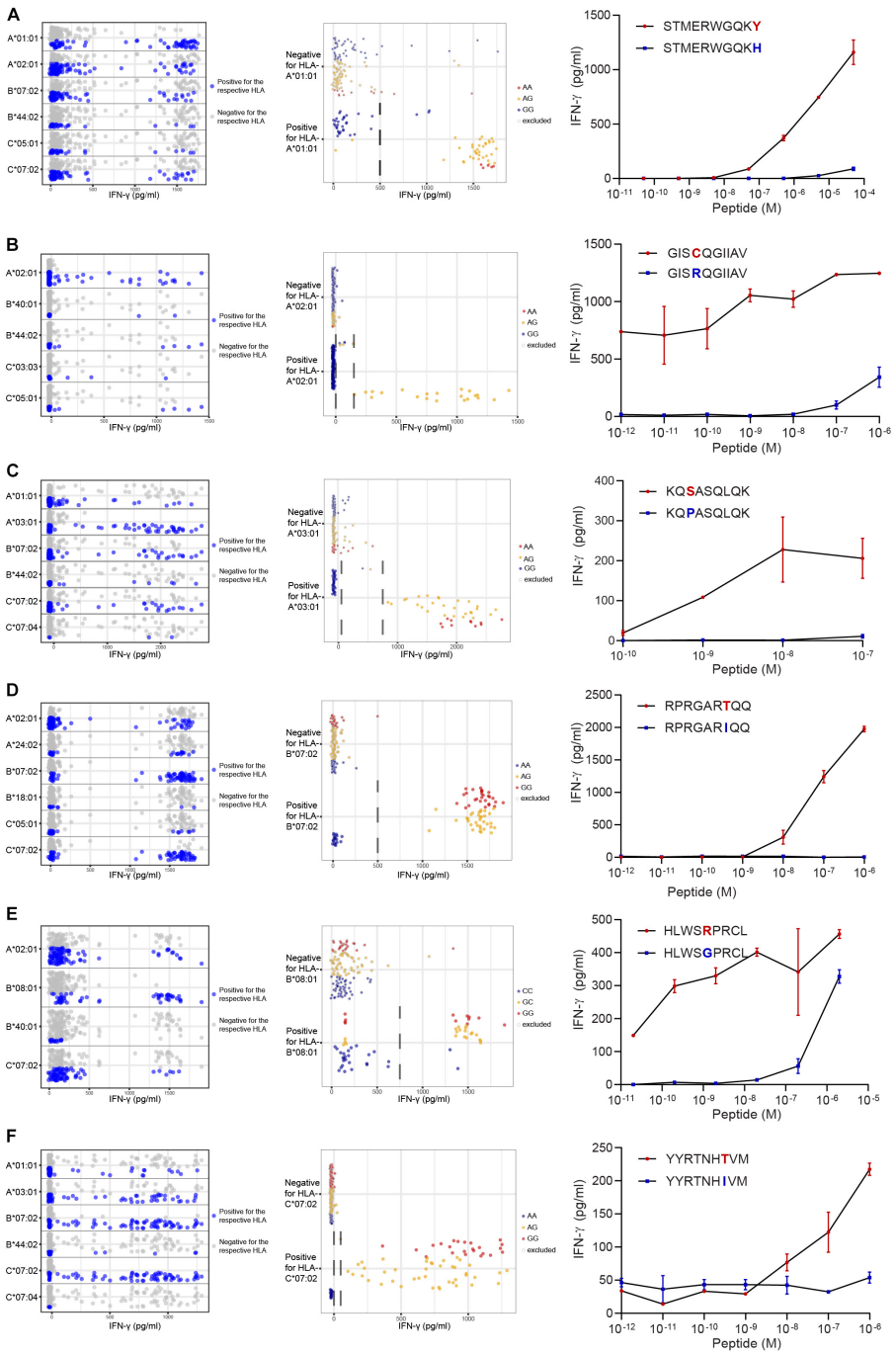


FIGURE 4 | Continued

FIGURE 4 | Identification of new minor histocompatibility antigens in common HLAs by optimized WGAs. T cell clones isolated from patients with hematological malignancies who responded to DLI after HLA-matched T-cell depleted alloSCT (**Table 2**), were tested for reactivity against 191 EBV-LCLs from the 1000 Genomes Project by IFN-γ ELISA. T-cell clones 14 **(A)**, 3B4 **(B)**, 2.1A12 **(C)**, 2–90 **(D)**, 4D8 **(E)** and H.9A6 **(F)** are shown. Data points show the IFN-γ release upon co-incubation with each of the 191 panel EBV-LCLs. First, HLA restriction was determined by analyzing EBV-LCLs that are recognized by the T cell clone for shared expression of one of the HLA class I alleles as expressed by the patient and donor (left graphs). For this purpose, the same dataset of IFN-γ values for 191 EBV-LCLs were separately displayed for each of the HLA class I alleles as expressed by the patient and donor. EBV-LCLs positive for the indicated HLA allele are shown by blue dots, while EBV-LCLs negative for this HLA are represented by gray dots. In a next step, EBV-LCLs are divided into positive and negative groups based on IFN-γ levels (indicated by dashed lines) while excluding those with intermediate IFN-γ levels (middle graphs) and WGAs is performed. SNPs that strongly associate with T cell recognition are analyzed for their distribution in EBV-LCLs that are positive and negative for the relevant SNP allele. EBV-LCLs that are homozygous or heterozygous for the associating SNP are represented by red and orange dots, respectively. EBV-LCLs that are homozygous for the allelic variant are indicated by blue dots. Gray borders represent EBV-LCLs that are excluded from the WGAs analysis based on intermediate IFN-γ levels or not expressing the relevant HLA allele. The middle graphs show results for rs4673 **(A)**, rs8069315 **(B)**, rs1050301 **(C)**, rs10749693 **(D)**, rs7080014 (rs7086691 not shown) **(E)** and rs1054487 **(F)**, which have been identified as associating SNPs by WGAs (**Supplementary Table S5**). The *p*-values of detection are shown in **Table 2** and **Supplementary Table S6**. Finally, coding regions surrounding associating SNPs were searched for peptides with predicted binding to the respective HLA restriction allele by NetMHC 4.0. Peptide candidates for potential minor histocompatibility antigens and their allelic variants were synthesized, titrated and pulsed on donor EBV-LCLs and tested for T-cell recognition by IFN-γ ELISA (right graphs). Indicated are peptide sequences for LB-CYBA-1Y **(A)**, LB-DHX33-1C **(B)**, LB-IMMT-1S **(C)**, LB-YIPF1-1T **(D)**, LB-STK32C-1R **(E)** and LB-MAN2B1-1T **(F)**, which have been validated as minor histocompatibility antigens.

TABLE 2 | Novel minor histocompatibility antigens identified by the optimized WGAs approach.

Patient	Disease	Immune Response	Clone	Antigen	SNP	HLA	AF[1]	Peptide[2]	Type of variant	Illumina 1M array[3]	Observed *p*-value
2877	CML	Conversion	14	LB-CYBA-1Y	rs4673	A*01:01	0.34	STMERWGQK[**Y**/H]	missense variant	y	8.21E-11
7010	MDS	GvHD	3B4	LB-DHX33-1C	rs8069315	A*02:01	0.11	GIS[**C**/R]QGIIAV	missense variant	y	9.61E-17
9528	MDS	GvHD	2.1A12	LB-IMMT-1S	rs1050301	A*03:01	0.33	KQ[**S**/P]ASQLQK	missense variant	n	7.22E-18
5177	AML	GvHD	2-90	LB-YIPF1-1T	rs10749693	B*07:02	0.67	RPRGAR[**T**/I]QQ	5 prime UTR variant	n	6.83E-16
3087	CML	Conversion	4D8	LB-STK32C-1R	rs7080014, rs7086691	B*08:01	0.33	HLWS[**R**/G]PRCL	missense variant	n	2.67E-06
9528	MDS	GvHD	H.9A6	LB-MAN2B1-1T	rs1054487	C*07:02	0.33	YYRTNH[**T**/I]VM	missense variant	y	8.11E-11
6711	AML	GvHD	B1	LB-CYBA-2Y/ LB-CYBA-3Y	rs4673	A*24:02/A*23:01	0.36	K[**Y**/H]MTAVVKLF	missense variant	y	7.95E-07

[1]Allele frequency in panel. [2]Amino acid change that leads to antigen recognition is presented in brackets in bold. [3]Part of Illumina Human 1M-duo SNP array.

as well as their allelic variants were subsequently pulsed on donor EBV-LCLs and tested for T cell recognition to validate minor histocompatibility antigens.

Following this strategy, we successfully identified minor histocompatibility antigens for seven T cell clones isolated from six patients (**Table 2**). Six of the seven new minor histocompatibility antigens are presented by HLA class I molecules for which the panel was designed. Of note, identification of LB-STK32C-1R as antigen recognized by HLA-B*08:01-restricted T cell clone 4D8 failed with our previous WGAs method, but succeeded with the new approach. This antigen is encoded by two SNPs causing a single amino acid change. Both SNPs were not measured on the 1.1M Illumina SNP array, but are included in the 1000 Genomes Project data files.

Peptide STMERWGQKY has been identified as LB-CYBA-1Y in HLA-A*01:01, whereas the same SNP also encodes peptide KYMTAVVKLF, identified as LB-CYBA-2Y. The latter peptide is presented by HLA-A*24:02 (**Figure 5A**), for which our panel has not been specifically designed. Although HLA-A*24:02 is expressed by only 29 EBV-LCLs of our panel, the SNP has an optimal allele frequency of 0.36, resulting in 17 EBV-LCLs that were recognized by the T cell clone and a *p*-value of 7.95×10^{-7} (**Figure 5B**). Another advantage of using a large panel of 191 EBV-LCLs is the possibility to identify antigens that are presented by more than one HLA restriction allele. For LB-CYBA-2Y, we noticed that six EBV-LCLs that lacked expression of HLA-A*24:02 were recognized by the T cell clone. Four of these EBV-LCLs were positive for the SNP for LB-CYBA-2Y and shared expression of HLA-A*23:01, an HLA molecule with a similar binding motif to HLA-A*24:02. By pulsing the peptide on K562 cells transduced with either HLA-A*24:02 or HLA-A*23:01, LB-CYBA-2Y was validated as minor histocompatibility antigen presented by two HLA-A molecules (**Figure 5C**).

In conclusion, we have improved the WGAs method for identification of minor histocompatibility antigens by selecting a new concise 1000 Genomes Project EBV-LCL panel

for seven common HLAs and demonstrated the value of this approach by successful discovery of seven novel minor histocompatibility antigens.

DISCUSSION

Identification of minor histocompatibility antigens is essential for improving the outcome of allogeneic stem cell transplantation. However, previous methods have been laborious and the number of identified antigens available for clinical use therefore remains limited. Here, we advanced current WGAs methods for discovery of minor histocompatibility antigens by designing a concise panel of 191 EBV-LCLs, which are sequenced as part of the 1000 Genomes Project, covering seven common HLA class I molecules.

For WGAs, CD8 T cell clones for unknown minor histocompatibility antigens were isolated from patients who had undergone alloSCT for treatment of hematological malignancies. These T cell clones were tested for reactivity against the new panel of 191 EBV-LCLs and subsequently screened for association with 10,955,109 biallelic SNPs and small indels with a minor allele frequency above 0.01. The genomic data do not contain gene deletions, but SNPs that are in strong linkage disequilibrium with common gene deletions (27) are included and can serve as markers for minor histocompatibility antigens encoded by these polymorphic genes, such as UGT2B17 (15, 17).

In our previous WGAs method, T cell reactivity was measured against a panel of 80 EBV-LCLs for which only 1.1 million SNPs were included on the Illumina SNP array (18). If the respective antigen-encoding SNP was not measured by the array, the antigen could only be indirectly identified via associating marker SNPs in linkage disequilibrium with the respective SNP. To evaluate the performance of our new panel of 191 EBV-LCLs, we selected T cell clones for five known minor histocompatibility antigens that are presented by different HLA class I alleles. Each of the five antigen-encoding SNPs was included in the list of strongly associating SNPs while only two SNPs were directly identifiable with the previous method. Likewise, for the seven novel minor histocompatibility antigens that were identified by the optimized WGAs approach, only 4 out of 8 SNPs were included on the 1.1 million Illumina SNP array.

The added value of the optimized WGAs strategy has also been confirmed by the discovery of LB-STK32C-1R, which is the target for an HLA-B*08:01-restricted T cell clone, for which previous WGAs had failed. T cell clone 4D8, which recognized LB-STK32C-1R at peptide concentrations as low as 10^{-11} M, showed also reactivity against its allelic variant at concentrations $>10^{-8}$ M (**Figure 4E**). Although clone 4D8 recognizes the allelic variant as exogenous peptide, it lacks reactivity against donor EBV-LCL, indicating that peptide presentation on the cell surface is not sufficient for T cell recognition when endogenously expressed. These data demonstrate that the TCR as expressed by clone 4D8 has a higher affinity for LB-STK32C-1R than for its allelic variant or, alternatively, that LB-STK32C-1R has a higher binding affinity for HLA-B*08:01. The latter possibility is supported by NetMHC 4.0 showing that LB-STK32C-1R is predicted to bind strongly, while its allelic variant is predicted to bind weakly to HLA-B*08:01. Furthermore, poor transportation by TAP, as reported for HA-8 (28) or proteasomal cleavage as demonstrated for HA-3 (29), may contribute to lack of recognition of the endogenous peptide.

Our optimized WGAs approach has been specifically designed to directly identify minor histocompatibility antigens in seven common HLAs without the need to retrovirally introduce these alleles for the European population. These common HLA molecules include HLA-A*01:01, A*02:01, A*03:01, B*07:02, B*08:01, C*07:01, and C*07:02. In the European population, 87.2% of individuals express at least one and 56.9% express two or more of the seven HLA alleles. The identification of new minor histocompatibility antigens in six of these HLA molecules confirms the adequacy of the panel size and design of the approach. For the seven HLAs for which the method has been specifically designed, minor histocompatibility antigens can be directly identified for SNPs with a wide range of allele frequencies. In addition, minor histocompatibility antigens can be directly identified for a number of HLA alleles that are less common, but only for SNPs within a more restricted range of allele frequencies. Our *in silico* analysis showed that ACC-1Y in HLA-A*24:02 (n = 29; number of EBV-LCLs expressing the HLA molecule) and ACC-1D in B*44:02 (n = 28) could be identified and also one of the new minor histocompatibility antigen, i.e., LB-CYBA-2Y, was shown to be presented by HLA-A*24:02. However, if the SNP has an allele frequency outside this restricted range, introduction of the HLA restriction allele into the panel of EBV-LCLs is necessary. Similarly, HLA alleles need to be introduced for all minor histocompatibility antigens that are presented by HLAs for which less than 25 EBV-LCLs are included in the panel. The HLA alleles and corresponding range of allele frequencies for SNPs encoding minor histocompatibility antigens that can be directly identified by our optimized WGAs method are shown in **Supplementary Table S3**. This table highlights the advantage of the new WGAs approach as compared to our previous method with 80 EBV-LCLs only expressing HLA-A*02:01 and B*07:02 and the method developed by Oostvogels et al. (20) using a panel of 43 EBV-LCLs from the 1000 Genomes Project in which the HLA restriction allele had to be introduced (**Figure 1**). Furthermore, the new WGAs method also allows for identification of antigens that are presented in more than one HLA as exemplified by LB-CYBA-2Y, which was found to be presented and recognized in HLA-A*24:02 as well as A*23:01. Presentation and recognition of the minor histocompatibility antigen in more than one HLA increases sample size, thereby enhancing the possibility to detect antigens in less frequent HLAs and expanding the range of SNPs with detectable allele frequencies.

As reported by Bykova et al. (30), SNPs with a high probability of mismatch between patient and donor have allele frequencies between 0.15 and 0.47. Our data showed that for seven common HLAs, all minor histocompatibility antigens encoded by SNPs with these allele frequencies can be detected with the new panel of EBV-LCLs from the 1000 Genomes Project. Only allele frequencies below 0.03 and above 0.73 corresponding to population frequencies below 0.06 and above 0.92, respectively,

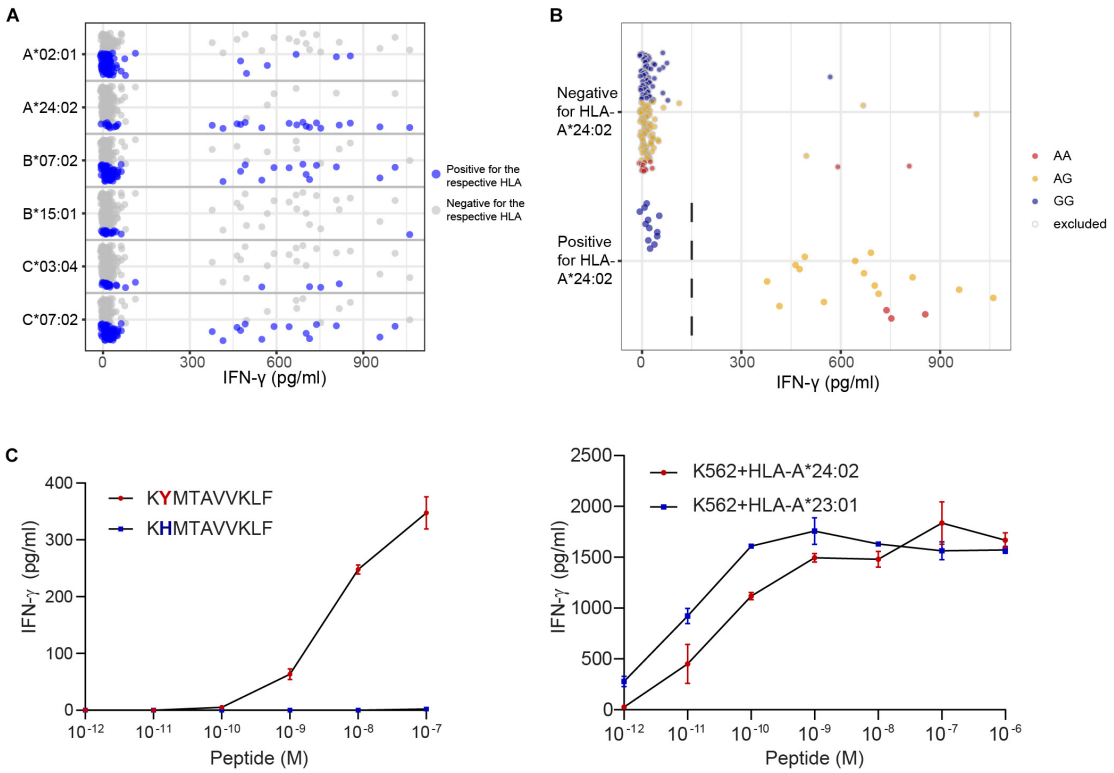


FIGURE 5 | Identification of a novel minor histocompatibility antigen in uncommon HLAs. **(A)** Clone B1 from patient 6711 appeared to be restricted to HLA-A*24:02, which is expressed by 29 EBV-LCLs in our panel. **(B)** Due to an optimal allele frequency of 0.36, missense SNP rs4673 could be identified by WGAs. Of note, 4 of 6 EBV-LCLs that lacked expression of HLA-A*24:02 were positive for rs4673 and shared expression of HLA-A*23:01. **(C)** Analysis of the region surrounding rs4673 revealed a peptide with strong predicted binding to HLA-A*24:02 and A*23:01. Pulsing the peptide on donor EBV-LCLs (left) and K562 cells (right) transduced with HLA-A*24:02 or A*23:01 confirmed T-cell recognition of LB-CYBA-2Y in both HLA class I alleles.

are outside of the predicted detection limits. The finding that all SNPs with a high probability of mismatch can be identified in seven common HLAs makes our optimized WGAs approach an ideal and more rapid strategy to identify the dominant repertoire of clinically relevant HLA class I-restricted minor histocompatibility antigens. Evidence that the repertoire of minor histocompatibility antigens is limited has been shown by Granados et al. (31) who predicted a maximum number of 50–100 antigens per HLA allele based on polymorphic peptides encoded by SNPs with a MAF of $\geq$0.05 that were identified for two HLAs on EBV-LCLs by mass spectrometry.

Due to high throughput sequencing techniques which enable genome wide detection of genetic variants, bioinformatic pipelines have been developed to predict neoantigens (32–35) and minor histocompatibility antigens (31, 36–40). Based on whole exome sequence data, Koparde et al. (39) found an average of 2463 non-synonymous SNP disparities in the Graft-versus-Host direction in patients transplanted with related donors, and an average of 4287 SNP disparities in patients transplanted with unrelated donors (39). SNP disparities in the same range have been reported by others (37). Martin et al. (40) showed that a higher number of SNP disparities in patients transplanted with sibling donors was associated with an increase in grade III-IV GVHD and stage 2–4 acute gut GVHD, whereas Ritari et al. (41) found an association between a higher number of mismatching peptide ligands and chronic GvHD. All SNP disparities as measured by Koparde et al. (39) were also investigated to encode 9-mer peptides with predicted binding to patients' HLA class I alleles using NetMHCpan 2.8. The results revealed 3670 peptides with intermediate and 852 peptides with strong binding in patients transplanted with related donors, and 5386 intermediate and 1160 strong binding peptides in patients with unrelated donors. Although minor histocompatibility antigens are probably present among these peptides, prediction tools are hampered by high false discovery rates due to failure to accurately predict intracellular HLA class I peptide processing. Whole transcriptome RNA sequencing and HLA ligandome analysis by mass spectrometry can be implemented as additional steps to select for peptides that are expressed and presented on the cell surface. These techniques significantly decrease false discovery (31, 38, 41, 42), but also reduce the sensitivity and lead to a higher chance that antigens are missed (38), illustrating that prediction tools for minor histocompatibility antigens still require optimization.

Whole genome association scanning is a technique that allows discovery of minor histocompatibility antigens with high sensitivity and specificity. The method is rapid and cost effective, since one panel of 191 EBV-LCLs can be used to identify antigens

for T cells from different patients. Another advantage is that discovery of minor histocompatibility antigens is not restricted to non-synonymous SNPs, but can also be performed for other polymorphisms, such as synonymous SNPs in alternative reading frames and intron SNPs in alternative splice variants. In order to perform WGAs, T cell clones are needed that are able to recognize EBV-LCLs. Since T cell responses after alloSCT may have been induced by professional antigen-presenting cells, there is a possibility that minor histocompatibility antigens exist that are myeloid specific which cannot be identified by our EBV-LCL panel. Furthermore, for antigens that are not encoded by SNPs such as neoantigens or Y chromosome encoded antigens, other techniques such as peptide (34) or mini-gene libraries (33) have to be employed.

Here, we optimized WGAs to enable discovery of the dominant repertoire of minor histocompatibility antigens in common HLA class I alleles. Discovery of this repertoire is relevant to predict and manipulate GvL and GvHD after alloSCT. Discovery of immunogenic antigens is also important to gain insight into the various cut-offs that need to be applied in prediction tools for minor histocompatibility antigens, which are particularly necessary to characterize antigens for HLA alleles and SNP mismatches that are rarer. As such, WGAs together with prediction tools may ultimately enable development of personalized strategies to separate GvL from GvHD, thereby improving clinical outcome after alloSCT.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ebi.ac.uk/arrayexpress/experiments/E-GEUV-1/files/genotypes/.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the LUMC Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KF, MH, EM, JF, and MG designed the research. KF, MH, EM, AA, DL, and MP performed the experiments. KF, MH, EM, AA, DL, MP, CB, JF, and MG analyzed the results. KF, RM, SK, and P'tH contributed to the bioinformatic analysis. JF and MG supervised the research. KF, SK, P'tH, CB, JF, and MG wrote the manuscript. All authors read and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00659/full#supplementary-material

FIGURE S1 | Regional origin of selected EBV-LCLs and common HLA distribution in continental groups of the 1000 Genomes Project. **(A)** The 191 selected EBV-LCLs from the 1000 Genomes Project are of different regional origin, including 176 (92.1%) of European origin (CEU: Utah Residents with Northern and Western European Ancestry, FIN: Finnish in Finland, GBR: British in England and Scotland, TSI: Toscani in Itay) and 15 (7.9%) belonging to individuals from Yoruba in Ibadan, Nigeria (YRI). **(B)** HLA typing was obtained from 2693 individuals in the 1000 Genomes Project to determine the frequencies of the seven common HLA class I alleles in different human subpopulations. After exclusion of 63 individuals with unclear subtypes, the occurrence in individuals **(B)** and population frequency **(C)** of HLA-A*01:01, A*02:01, A*03:01, B*07:02, B*08:01, C*07:01, and C*07:02 was counted for individuals of European (EUR, *n* = 521), admixed American (AMR, *n* = 363), South Asian (SAS, *n* = 522), African (AFR, *n* = 697) and East Asian (EAS, *n* = 527) background.

TABLE S1 | HLA co-expression in the EBV-LCL panel.

TABLE S2 | In silico analysis of published antigens.

TABLE S3 | Detectable ranges of minor allele frequencies.

TABLE S4 | SNP associations for clones recognizing known minor histocompatibility antigens.

TABLE S5 | Genotyping for EBV-LCL panel of antigen-encoding SNPs.

TABLE S6 | SNP association of new minor histocompatibility antigens.

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OPINION
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Can Graft vs. Leukemia Effect Be Uncoupled From Graft vs. Host Disease? An Examination of Proportions

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Hematopoietic cell transplantation (HCT) provides potentially curative therapy for high-risk hematological malignancies, predominantly through alloreactivity mediated by donor immune effectors directed at a recipient's malignant cells; this is termed graft vs. leukemia (GVL) effect (1). This beneficial effect has historically been associated with a similar donor immune attack on normal recipient tissues, graft vs. host disease (GVHD) (2). At this time both of these entities, GVL and GVHD are considered to be stochastically determined, i.e., prior to transplant one cannot reliably determine which patient will develop one or both outcomes. The high complexity of the system at hand which includes patients with different malignancies, varying human leukocyte antigen (HLA) types, and different immune effectors involved in these processes has meant that logic-based therapeutic choices which impact variables associated with GVL are studied by determining the probability of the desired clinical outcomes in large populations of patients. Such studies have allowed an incremental improvement in the clinical outcomes of recipients of similarly HLA matched donor HCT. The introduction of high-resolution HLA matching and HLA DPB1 matching were both such incremental changes which helped improve survival in recipients from HLA matched HSC donors (3–5). Despite these advances, the apparent randomness in the potential for developing alloreactivity remains. This apparent randomness derives, in part from these phenomena having their origin at a molecular level, with the recognition of minor histocompatibility antigens (mHA) and tumor specific antigens (TSA) bound to HLA molecules on the antigen presenting cells (APC), by unique T cell receptors (TCR) on T cell clones. This recognition triggers T cell responses which effect the observed clinical outcomes.

To develop a deeper understanding of the alloreactive processes governing the relative balance of GVHD and GVL one has to understand the antigenic landscape at hand in a HCT recipient at the molecular level. Herein is presented a model which examines the relative difference in the genetic *potential* for developing either GVL, using tumor specific antigen (TSA) burden, or likelihood of developing GVHD, using minor histocompatibility antigens (mHA). Historically haematopoietically restricted mHA (6–8), cancer testis antigens (9), protein splice variants (10) and in some instances even retroviral elements (11) have been implicated in producing GVL effects, with some of these elements also contributing to GVHD. While haematopoietically restricted mHA have been implicated in the development of GVHD and protection from relapse (12), HLA presentation is a prerequisite for this to occur (13). Thus, far ∼60 haematopoietically restricted minor histocompatibility antigens have been described with antigen presentation restricted to a limited spectrum of HLA allotypes, precluding broad utility in patients (7). Thus, to optimize clinical outcomes, it is imperative to develop

methodology which will allow personalized computation of the probability of GVHD or GVL developing in unique HCT donor-recipient pairs.

Hematological malignancies are driven by DNA mutations which develop in normal cells over time as a result of exposure to external mutagens and intrinsic processes, such as errors in DNA replication (14). The mutational burden of adult cancers ranges widely; for example, solid tumors may average from 33 to 66 somatic mutations which alter their protein structure and function. Cancers such as, melanoma and lung cancer are on an extreme end of this spectrum, possessing ∼200 mutations per tumor, and thus are susceptible to immune therapy (15). On the opposite end of this spectrum, hematologic malignancies have some of the lowest mutational burdens, with leukemias harboring ∼9.6 mutations per tumor (14, 16–20). Mutated genes expressed in these tumors may be recognized as non-self-proteins by the immune system, and targeted by the GVL mechanism (21).

Point mutations were first shown to induce a naturally occurring T cell response in a patient with melanoma (22). However, initial studies of cancer immunotherapy were hampered by technological challenges encountered in deriving patient specific TSA libraries. In the past decade, next generation sequencing (NGS) or "deep" sequencing has allowed the sequencing of thousands of small fragments of DNA in parallel, such that an entire genome may be rapidly sequenced (23). NGS has allowed cataloging of the entire library of potential TSA in a variety of human malignancies. The full impact of this knowledge of individualized genetic profiling of cancers was first observed when utilizing programed death receptor PD-1 and programed death-ligand (PDL) receptor inhibitors. Check point blockade allows unimpeded autologous TSA specific T cell mediated killing, which is most significant in tumors with a higher mutational burden, as there are theoretically more TSA presented on MCH class I and class II molecules with a greater mutational burden (14, 24, 25).

In contrast, the relatively low mutational burden of hematologic malignancies does not meet the thereshold necessary to effectively utilize immunotherapy and PD1/PDL1 blockade (26, 27). Common hematologic driver mutations including NPM1 which are expressed in 30–35% of cases of AML have been shown to be expressed by AML blasts and may be targeted by TCR gene transfer (28). Several other specific mutations including BCR-ABL, WT1, and PR1 have also been shown to effect outcomes after HCT (29–31). Nevertheless, such unique mutations are usually not adequate to generate an intrinsic GVL response in the vast majority of patients. Despite this relative dearth of tumor associated neo-antigens, hematological malignancies have proven to be susceptible to the GVL effect of an allograft, some times without GVHD developing (32–34). One may therefore ask, is it possible to apply NGS to the transplant setting in order to understand how one may uncouple GVL from GVHD in the majority of patients? This goal has been sought by many a group who have tried to better predict GVHD and GVL by examining biomarkers (35), cytokines (36, 37), mass spectrometry data (38), natural killer cell markers (39). Modification of the conditioning and GVHD prophylaxis regimens have also been attempted to accomplish the dissociation of GVHD from GVL (40–43). However, while all of these factors play important roles in the GVHD and GVL phenomenon, if both at their core are centered on peptide presentation and immune attack, it is not likely that we can always dissociate GVL from GVHD.

A computational approach may be taken to develop a partial understanding of the GVHD-GVL balance in HLA matched HCT. As stated above, on average hematologic malignancies contain ∼10 protein coding, exomic mutations which may be immunogenic. For patients with these and other TSA resulting from mutations, logically in each individual, the number of tumor specific peptide antigens presented will then depend on their HLA type, the specific mutations and the spectrum of mutated peptides presented by those HLA molecules. As an example, a study of over 600 patients with multiple myeloma showed an average of 64 nonsynonymous mutations. Neoantigen load was then predicted *in silico* by identifying mutant peptides predicted to bind class I HLA molecules. Predicted neoantigen were defined as any unique peptide: HLA combination with mutant binding affinity IC50 less then 500 nM. This revealed the average predicted neoantigens to be 23 in number, with 9 expressed neoantigens. This outlines the fact that not all neoantigens are either expressed or presented on HLA (17). This number then gives an approximate estimate of the isolated GVL inducing potential for multiple myeloma. However, the average number of nonsynonymous mutations in leukemia is typically much lower, as noted above. If we were to extrapolate using the ratio of 64 nonsynonymous mutations to its 9 expressed neoantigens, one could predict that hypothetically hematologic malignancy on average would be unlikely to express >10 neoantigens. In actual fact the true number of TSA will vary with each individual based on the number of nonsynonymous mutations present, type of mutation (i.e., point vs. frame shift mutations) their antigenicity, cleavage potential of the proteins harboring the mutations, the HLA binding affinities of the mutant peptides and the HLA type in an individual, among other factors. While, this may underestimate of the expressed neoantigens burden of hematologic malignancy, a study of antigen presentation of multiple malignancy types including hematologic malignancies and solid tumors indicated that there are ∼1.5 expressed neoantigens per point mutation and 4 per frameshift mutation (44), suggesting that the estimate presented here is not too far from reality.

This may hold true even if one considers other TSA sources that may contribute to GVL, including those derived from normally repressed proteins such as cancer testis antigens. These are antigens normal expressed in "immunologically privileged sites" such as, testicular or trophoblastic tissues, and are thus immunogenic. When expressed, these will offer a potential GVL target, which will not be dependent on TSA, and will add to the TSA burden. However, there is variability introduced at the response end of this cascade, since some of these mutations may lead to too strong a TCR affinity and down regulation by central tolerance, while others with a more optimal affinity being allowed to escape central tolerance while still allowing allowreactivity

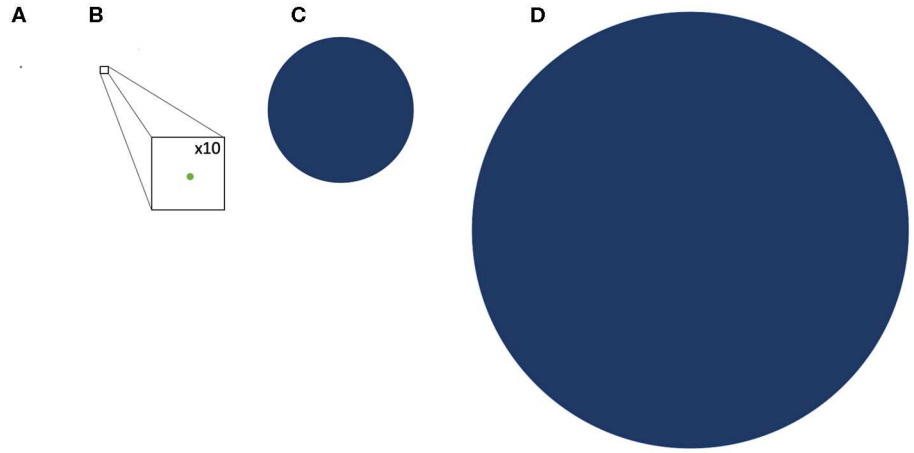

FIGURE 1 | Comparative representation of the TSA and GVL potential of hematologic malignancy and GVHD drawn to scale. **(A)** representation of the average mutational burden of hematologic malignancy (9 SNPs) **(B)** A representation of the TSA, the potential peptide presentation of hematologic malignancy mutations in HLA class I; GVL Burden (drawn with x10 magnification to make visible) **(C)** representation of the potential DRP minor histocompatibility antigen proteins loaded onto HLA class I; GVHD burden **(D)** representation of the average SNP burden between DRP resulting from nonsynonymous polymorphisms.

(45). All in all, it is unlikely that most hematological malignancies have a very large abundance of TSA to drive an isolated GVL phenomenon.

With an estimate of the TSA in hematological malignancies established, one may next attempt to determine how likely it is to unravel GVL from GVHD. NGS also offers a perspective into the genetic background of GVHD alloreactivity. Exome sequencing in both hematopoietic stem cell as well as solid organ transplant recipients has demonstrated a vast library of potential mHA which provide an alternative set of targets for donor T cells. Whole exome sequencing (WES) of transplant donors and recipients was performed in a group of HLA matched donors and recipients, and demonstrated an average of >6,000 non-synonymous single nucleotide polymorphisms (SNP) per HLA matched donor-recipient pair (DRP) (46). These polymorphisms when translated into peptide sequences *in silico*, yielded an average of 2,254 peptides/DRP with the potential to bind HLA-A, -B and -C molecules with intermediate to high affinity (IC50 of <50 nM, NetMHCpan ver2.0) (47) and represented an alloreactivity potential for a given HSCT DRP. The SNPs when compared to the mutations used to estimate TSA, are much larger in number, indicating that mHA may provide the dominant antigen background in terms of generating alloreactivity following HCT. Similar data regarding the extent of genomic variation between transplant donors and recipients have been reported by other groups investigating genomic variation in transplant recipients, in both solid organ transplants (48) and in HCT (49–53), as well as in models predicting GVL specific libraries (54). This abundance of SNPs across the exome in unique HCT donor-recipient pairs is an eye-opening finding compared to the average 10 mutations per hematologic neoplasm. This relative antigen abundance of potential mHA compared to the potential TSA estimate is graphically depicted to scale in **Figure 1**.

While the sheer number of mHA alone vastly outnumbers the potential TSA in hematologic malignancy, these numbers do not tell the whole story. Whether the potential mHA result in a T cell proliferation depends on several factors, such as peptide cleavage potential, antigen binding affinity, and critically, T cell clones bearing receptors that might recognize the mHA-HLA complexes. Crucially, the T cell receptor affinity for HLA-mHA or HLA-TSA complexes also needs to be adequate to ensure T cell engagement and activation. Mathematical modeling of T cell expansion in response to these HLA-antigen complexes has given important insights into the quantitative principles at hand in these processes. First, the expansion of donor T cells recognizing specific antigens will be proportional to the amount of antigen available, i.e., the expression level of the antigen bearing protein will determine the extent of T cell expansion. Secondly, this T cell expansion is likely governed by the affinity of the antigen to the HLA molecule, and the affinity of the T cell receptors for antigen-HLA complex. This is an exponential relationship, with T cell growth increasing non-linearly in response to changing affinity. An important clue to this is provided by the T cell clonal frequency distribution which follows Power Law when these are plotted out for T cell clones present in normal individuals (55).

$$T\ cell\ frequency \propto Antigen\ expression \times e^{mHA-HLA\ affinity \times TCR\ affinity}$$

Based on the above model, an alloreactive donor cytotoxic T cell response was simulated. To do this the array of mHA in each patient was considered as an operator matrix modifying a hypothetical cytotoxic T cell clonal vector matrix. Utilizing the basic assumption that T cell expansion will be governed by the binding affinity of the variant peptide to HLA, and for model estimation of antigen driven T cell proliferation, assuming unit affinity of the TCR for each mHA-HLA complex (since this was not known for this particular set of antigens), each responding T

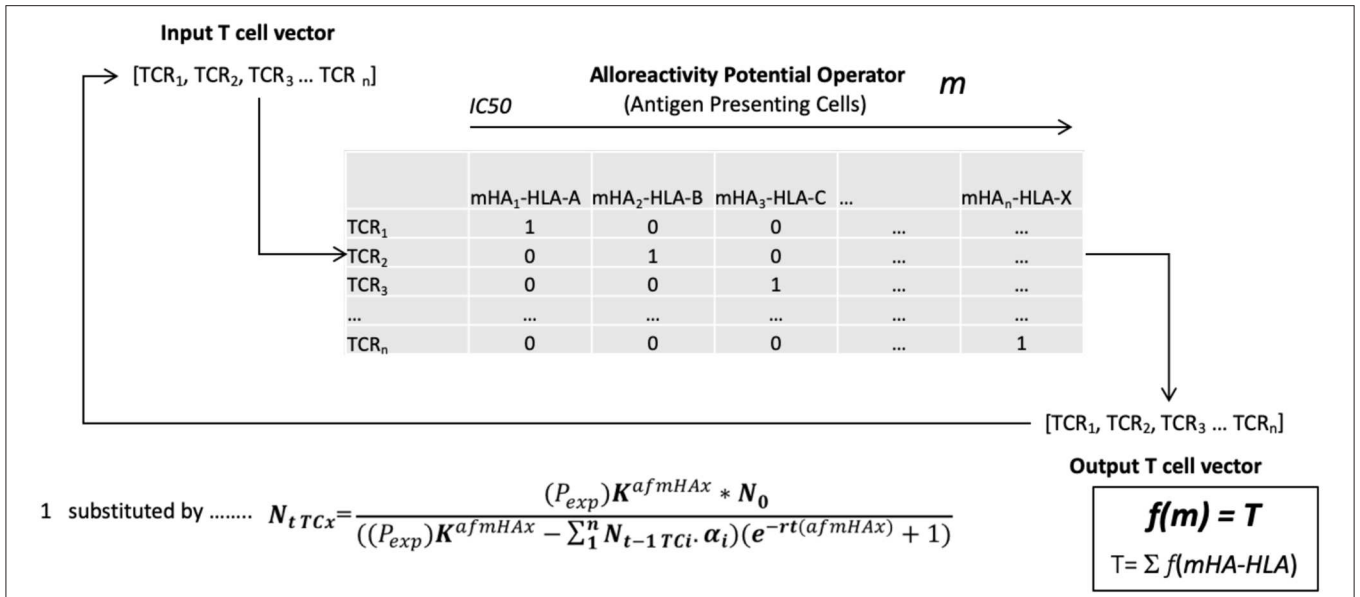

FIGURE 2 | The vector operator model of minor histocompatibility antigen presentation to T cell receptors in an individual. TCR_i- i^{th} T cell receptor; mHA_i-HLA- i^{th} minor histocompatibility antigen-HLA complex; Nt_{TCx}- Number of T cell at time t; P_{exp}- polymorphic protein expression; K- Growth constant; N_0- Starting T cell count; afmHA- Affinity of mHA*TCR affinity; *r*- Growth rate; *m*- cumulative mHA-HLA burden, alloreactivity operator; *T*- Total simulated T cell vector.

cell clone's proliferation was determined by the logistic equation of growth (**Figure 2**). Assuming uniform growth conditions, *r* values in the logistic equation, these simulations, showed that the *simulated* organ-specific alloreactive T cell clonal growth had marked variability, with orders of magnitude of difference between different HLA matched DRPs ($N = 78$). This was because of the differences in the unique polymorphic peptide sequences and their binding to the many different HLA types. In this study higher total and organ-specific T cell counts were associated with the incidence of moderate to severe GVHD (56). T cell growth in these simulations exhibited a sigmoid, logistic dynamic over time similar to immune reconstitution kinetics exhibited by allograft recipients (57). This model predicted the emergence of a limited number of dominant T cell clones responding to highly expressed and high affinity mHA—HLA class I complexes unique to each individual depending on their HLA type. On the other hand, there was a large number of low frequency clones responding to poorly expressed protein-derived mHA, weakly bound to the corresponding HLA. When the model was adjusted to incorporate competition with dominant higher affinity clones, it demonstrated chaotic dynamics with suppression of the lower affinity clones in early time points, identifying this as a possible contributor to the stochasticity observed in the clinical setting. Further, once variability in TCR affinity for the mHA-HLA complexes is accounted for in this model, then the even greater variability and randomness in T cell responses may be observed between different donor-recipient pairs. Change in the term for growth rate, *r* in the model will have profound impact on the variability seen and GVHD risk. When evaluated for HLA class II molecule presentation, these alloreactive mHA libraries further expanded several-fold given the longer peptide sequences which may bind HLA class II molecules, increasing the mathematical complexity at hand. Nevertheless, this work demonstrates that these antigen arrays are susceptible to mathematical modeling and thus of potential use in estimating the likelihood of GVHD occurring in HLA matched (or mismatched) SCT DRP (58). Such estimates will potentially serve to personalize GVHD prophylaxis regimens to allow optimal GVL effect in future trials, while suppressing GVHD.

With these data in mind, when the relative number of tumor specific antigens and minor histocompatibility antigens are examined it becomes obvious that the relatively small number of TSA compared with mHA, may in most individuals result in outcompeting of tumor specific targets, by normal tissue targets setting up the field for GVL occurring in the company of GVHD (**Figure 1**). Thus, polymorphic normal recipient antigens (mHA) expressed in the malignant clones will be more likely to be presented to the donor T cells and contribute to a relapse-free-state, than TSA. The mathematics are further complicated by the possibility that the TSA compete not only with the mHA for presentation, but also with the non-polymorphic/non-antigenic peptides in the recipient's tissues, which will far outnumber both these sets of peptides, since these will also be loaded onto the HLA molecules and presented to the donor helper and cytotoxic T cells. The mathematics dealing with this problem were introduced in the paper by Salman et al. It is also imperative that the immunogenic antigens have peptides with an affinity to both HLA class I and HLA class II molecules and be expressed in a particular malignancy in an individual for those to be effective at provoking an immune response.

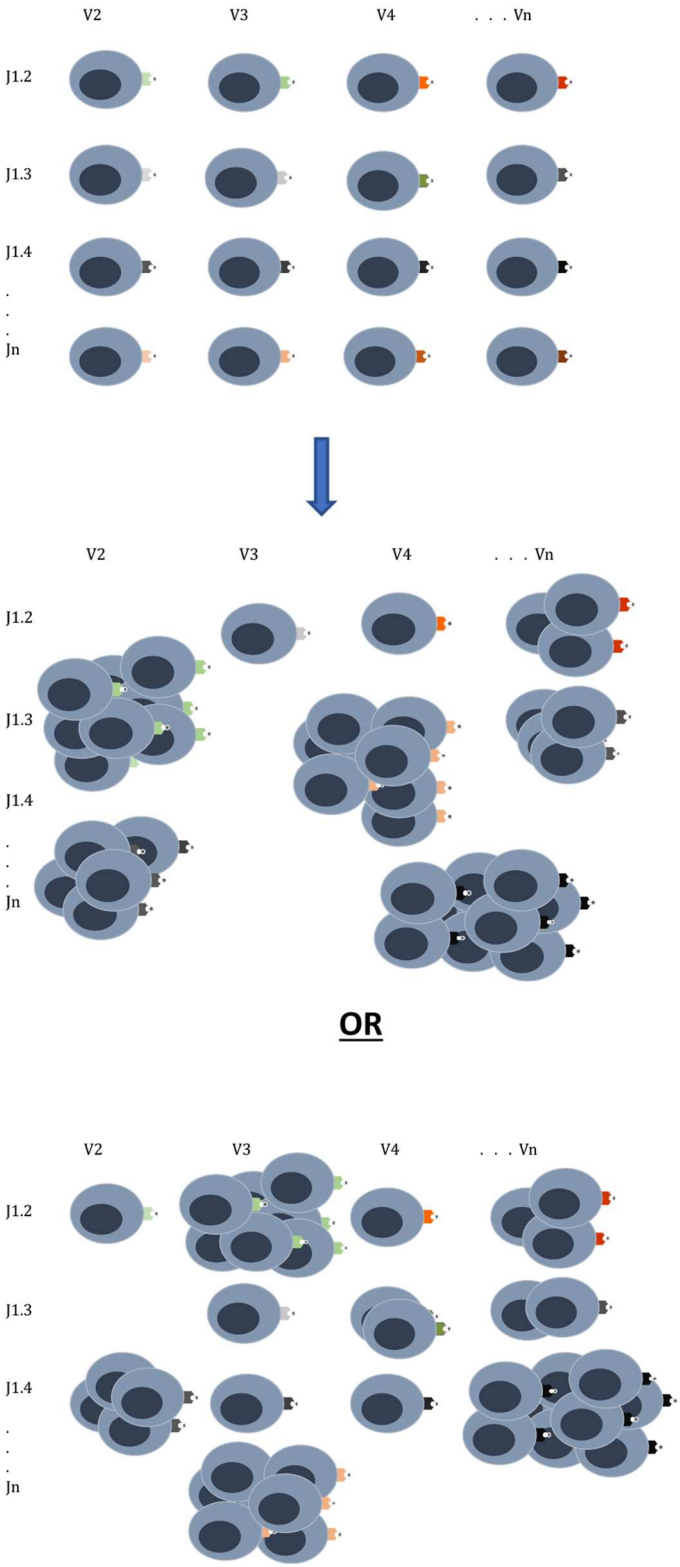

FIGURE 3 | T cell clonal proliferation will depend on affinity and abundance of antigen at the time of initial exponential expansion. The donor graft has a T cell clonal repertoire with the potential to react to many different antigens. Once infused into the recipient the T cell clones expand in proportion to the relative antigen affinity and abundance as can be seen in two different scenarios emerging from the same donor cell infusion. Other factors which will influence this growth are cytokines, degree of tissue injury and pharmacotherapy for GVHD prophylaxis.

All is not lost in the mathematical medley of chaos and combinatorics. It is clear that the quantitatively driven T cell responses depend on relative antigen abundance and HLA affinity. Traditionally HSCT is done with patients in remission, and as immunosuppression is withdrawn, they may develop chronic GVHD, which confers protection from relapse, and in a few patients GRFS might be observed. This likely depends on both the extent of T cell clonal diversity emerging after transplantation, as well as the balance of antigen expression. It is therefore critical to understand the notion of relative antigen abundance (**Figure 3**), such that to elicit an effective immune response an antigen has to be present in an adequate quantity. Such relative antigen abundance of TSA and mHA may be modulated by vaccination using TSA, as has been reported in melanoma patients (59). This may increase the likelihood of GVL developing in a GVHD-free state in patients with hematological malignancies. It is important to recognize the logistic growth kinetics of T cell clones with an early exponential growth phase, and the importance of timing in vaccine administration before the onset of this growth. Another approach already in practice is to use hypomethylating agents to alter the expression of immunogenic cancer testis antigens (9). This therapy provides an extensive library of alternative immune targets for the donor T cells to focus on and has been successfully combined with donor lymphocyte infusions to treat post allograft relapse (60). It is to be recognized that this model only partially encompasses the complexity of normal and post-transplant immune responses and does not give a complete explanation for the GVHD-GVL dissociation observed in patients who experience GRFS, That state represents a complex interplay of the factors described here with conditioning regimens and GVHD prophylaxis, and of course tumor growth kinetics. Antigen presentation triggered by tissue injury and cytokine release are critical factors in these calculations, as are pharmacological suppression of T cell growth, and elimination of T cell clones.

In conclusion, mathematical modeling of immune reconstitution, guided by NGS, along with an in-depth analysis of the relative expansion of donor T cell clones in response to the differentially expressed TSA and normal recipient antigens in individual patients, may allow a deeper understanding of the apparently stochastic nature of clinical outcomes observed at a population level. Mathematical modeling of T cell responses has revealed the chaotic dynamics of post-transplant immune responses, when multiple antigens with different HLA binding affinities and tissue expression levels are studied (58, 61, 62). Thus, stochasticity is built into the system, however, the probability windows for GVHD-GVL determination, may be narrowed by a using tools such as NGS of normal and malignant recipient, as well as donor exomes, and mathematical simulation of alloreactive T cell responses to mHA and TSA. These strategies can be used to identify the optimal TSA which would yield a T cell response, and these may then be used to derive tumor specific vaccines, altering the relative antigen abundance at crucial early times following SCT. Thus, in-depth genomic analysis may eventually allow us to truly develop precision medicine tools for optimizing patient outcomes following SCT.

AUTHOR CONTRIBUTIONS

EK and AT developed the idea and wrote the manuscript.

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H60: A Unique Murine Hematopoietic Cell-Restricted Minor Histocompatibility Antigen for Graft-versus-Leukemia Effect

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an important treatment for many types of hematological malignancies. Matching of donor and recipient for the major histocompatibility complex (MHC) improves the HSCT reconstitution, but donor-derived T cells reactive to non-MHC encoded minor histocompatibility antigens (MiHAs) can induce graft-versus-host disease (GVHD) while also being needed for graft-versus-leukemia (GVL) effects. MiHAs are allelically variant self-peptides presented conventionally on MHC molecules, but are alloantigenic in transplantation settings. Immunodominant MiHAs are most strongly associated with GVHD and GVL. There is need for mouse paradigms to understand these contradictory effects. H60 is a highly immunodominant mouse MiHA with hematopoietic cell-restricted expression. Immunodominance of H60 is tightly associated with its allelic nature (presence vs. absence of the transcripts), and the qualitative (TCR diversity) and quantitative (frequency) traits of the reactive T cells. The identity as a hematopoietic cell-restricted antigen (HRA) of H60 assists the appearance of the immunodominace in allo-HSCT circumstances, and generation of GVL effects without induction of serious GVHD after adoptive T cell transfer. Also it allows the low avidity T cells to escape thymic negative selection and exert GVL effect in the periphery, which is a previously unevaluated finding related to HRAs. In this review, we describe the molecular features and immunobiology in detail through which H60 selectively exerts its potent GVL effect. We further describe how lessons learned can be extrapolated to human allo-HCST.

Keywords: H60, minor histocompatibility antigen, graft-versus-leukemia, graft-versus-host-disease, hematopoieic cell-restricted antigen

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) was originally developed as a means to reconstitute the immune system of patients with hematological malignancies after anti-tumor radio/chemotherapy (1). T cell repopulation shortly after transplantation is attributed to the expansion of mature T cells from the donor bone marrow (BM) inoculum, rather than *de novo* T cell regeneration (2). Ideally, these mature donor-derived

T cells confer rapid protection from infection following allo-HSCT, while also being cytotoxic to residual tumor cells. This latter phenomenon is referred to as the graft-versus-leukemia (GVL) effect (3). Thus, allo-HSCT is considered as an anti-tumor treatment modality beyond its immune reconstitution capability. Mechanistically, donor-derived mature T cells elicit the GVL effect via recognition of host allo-antigens expressed by hematopoietic tumor cells (4). The downside is that they can also attack normal host tissues expressing allo-antigens and induce severe systemic inflammation, multi-organ failure, and mortality, a syndrome referred to as graft-versus-host disease (GVHD) (5). Although major histocompatibility complex (MHC)-matched transplantation significantly reduces the risk of GVHD, disparity at minor histocompatibility antigens (MiHA) continues to incur risk for GVHD whose target organs include intestine, skin, and liver (5–7). Thus, a matter of great interest is to minimize GVHD, while retaining the anti-tumor response. Particularly strong MiHAs whose expression is limited to hematopoietic cells are attractive targets for accomplishing this goal.

MiHAs arise from the fraction of self-peptides presented conventionally on MHC molecules that happen to be allelically variant (8). Their antigenicity is revealed in transplantation settings because such variant peptides are perceived as foreign to a host's T cells. With the advances in genome wide sequencing and T cell-epitope identification technologies, the number of molecularly identified MiHAs has increased exponentially (9–11). Immunodominant MiHAs have attracted attention as immunotherapeutic targets for hematologic malignancies (12–14). In this review, we describe the molecular features and immunobiology of an unusually immunodominant mouse MiHA, H60, that engender its potent GVL effect.

H60 AND ITS IMMUNODOMINANCE

Many of mouse MiHAs were identified at the molecular level in the late 1990s and early 2000s (8). Of these, MiHAs for which the specific T cell responses have been functionally evaluated are listed in **Table 1** (15–25). Although MiHAs are short peptides processed from various proteins, the molecular functions of the native proteins are in general irrelevant to their ability to generate allo-responses. Prototypic MiHA-specific allo-responses emanate from sequence variation within their MHC-presented peptides. The MiHA H60 differs in two respects. First, the native H60 protein serves as a ligand for the NK cell receptor NKG2D (26, 27). However, this function is unrelated to the role of H60 as a MiHA (H60 family proteins are introduced in **Box 1**). More importantly, H60 differs in that its allogenicity is based on its presence or absence of the transcripts (*H60*^{C} or *H60*^{null} allele; ^{C} represents allelic variant C, and ^{null} represents alleles with no transcripts) (15). Thus, T cells developed in C57BL/6 (B6; H-2^{b}) mice, which have the *H60*^{null} allele and, thereby, do not express H60, become activated when they encounter the completely foreign H-2K^{b}-LTFNYRNL peptide (H60p) processed from the protein produced by mouse strains carrying the *H60*^{C} allele, such as BALB and 129.

In a B6 vs. BALB.B pair, a representative example of MHC (H2^{b})-matched allogeneic donor and recipient mouse strains, MiHA number has been estimated up to 88 (29). However, the immunodominance phenomenon focuses the immune responses to fewer antigens, thus simplifying the complexity of the allo-response. Four MiHAs (H60, H4, H28, and H7) account for great majority of the B6 CD8 T cell responses to allogeneic BALB.B cells (30). But H60 stands out in that it accounts for more than 30% of the B6 anti-BALB.B allo-response (**Table 1**). H60-specific CD8 T cells expand up to 12% of the CD8 T cells in the blood of B6 mice once immunized with BALB.B splenocytes [this is termed B6 anti-BALB.B host-versus-graft (HVG) response] and compete effectively with CD8 T cells for the allo-MHC (H-2^{d}) proteins during the B6 anti-haploidentical CB6F1 HVG response (30, 31). H60 immunogenicity is even more intensified in GVHD. The frequency of the H60-specific CD8 T cells surges up to 25% of CD8 T cells in peripheral blood and target organs of BALB.B GVHD hosts (7). H60-specific CD8 T cells also prevail in other H2^{b}-matched GVHD pairs, such as B6 BMT to A.BY, LP/J, and 129 strains. This unusual level of immunodominance endows the value of H60 as a model MiHA to manipulate GVH and GVL responses, with growing evidence favoring the uniqueness of H60 as a GVL target, and is discussed subsequently.

HOW AND WHEN IS H60 IMMUNODOMINANT?

Hematopoietic Cell-Restricted Expression

Most known MiHAs exhibit ubiquitous expression patterns. However, H60 is only expressed by hematopoietic lineage cells in mouse strains carrying the *H60a*^{C} allele (15, 27). *H60a* transcripts are detected in lymphoid organs including the thymus and spleen, but not in the kidney, brain, and intestine of BALB/c mice (28, 32). Although one report claimed that *H60a* transcripts were found at appreciable levels in some non-hematopoietic tissues such as cardiac and skeletal muscles and skin (28), its expression in non-hematopoieic parenchymal cells has not been validated in allogeneic solid tissue (skin or heart) transplantation models (as will be described below), and could not be confirmed in our laboratory.

In general, H60-specific CD8 T cells undergo robust expansion, attaining peaks of 10–15% of blood CD8 T cells, when B6 mice are immunized with splenocytes from H60 congenic mice (B6.CBy-*H60a*^{C}; Con-H60 hereafter) (33–35). However, when tail skin from the Con-H60 strain is transplanted onto B6 mice (Con-H60→ B6), minimal specific T cell expansion is observed (to an average peak of 3%) (36). Similarly, Con-H60→ B6 skin or heart transplantation is associated with minimal skin graft rejection or coronary artery vascular disease (37). This contrasts greatly with the serious complications (almost 100%) found after skin or heart transplantation when the H60-mismatched donor is the H60 transgenic mouse line, C57BL/6 Tg (ACTB-H60a*) in which H60 is ubiquitously expressed under the control of the actin-promoter (termed Act-H60 Tg, hereafter) (37, 38). Similarly high rates of complications are observed when the solid tissue transplantations feature

TABLE 1 | Mouse minor histocompatibility antigens.

Name	Distribution	MHC ($H2^b$)*	C57BL/6 (allele/or X) sequence	BALB.B (allele/or Y) sequence	Proportions in B6 anti-BALB.B MLC (30)	References
H60 (*H60a*)	Hematopoietic	K^b	(b) Null	(c) LTFNYRNL	29.1–36.3%	(15)
H4 (*Emp3*)	Broad	K^b	(a) SGTVYIHL	(b) SGIVYIHL	6.5–26%	(16)
H28 (*IFi44l*)	Interferon-induced	K^b		(b) ILENFPRL	6–24.3%	(17)
H7 (*H7*)	Broad	D^b	(a) KAPDNRETL	(b) KAPDNRDTL	5–8%	(18)
H3a (*Zfp106*)	Broad	D^b	(a) ASPCNSTVL	(a) ASPCNSTVL		(19)
H13 (*H13*)	Broad	D^b	(a) SSVVGVWYL	(b) SSVIGVWYL	1–4%	(20)
HY-*Uty*	Broad	D^b	(X:*Utx*) WMHHTVDLL	(Y) WMHHNMDLI	2–2.5%	(21)
HY-*Dby*	Broad	A^b	(X:*Dbx*) SSSFSSSRASSSRSG	(Y) NAGFNSNRANSSRSS		(22)

*Underline, amino acid variation between strains. *Superscript b indicates b haplotype of H2.*

BOX 1 | The native function of H60.

In terms of molecular function, the native H60 protein serves as a ligand for the NK cell receptor NKG2D (26, 27). After paralog genes (*H60b* and *H60c*) encoding additional NKG2D ligands were identified (28), the original *H60* was renamed *H60a*. However, *H60b* and *H60c* encode proteins exhibiting amino acid variations at multiple sites including the H60p sequence, LTFNHRTL and LTVKYRTL, respectively, and were found to be transcribed in both the B6 and BALB strains (28). Thus, the MiHA H60 (simplified to H60, hereafter) refers to only the *H60a*-encoded protein that induces the potent T cell allo-response in mouse strains with the $H60a^{null}$ allele (eg., B6).

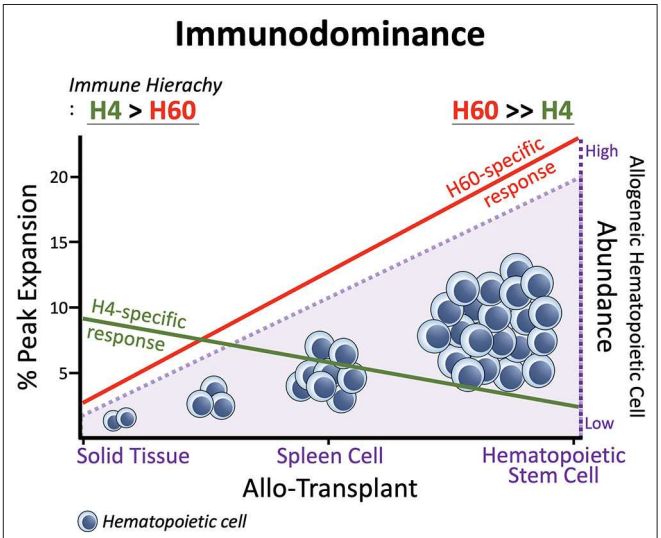

FIGURE 1 | Immunodominance of H60. Expansion of H60-specific CD8 T cells is promoted as the allogeneic hematopoietic cell abundances increase in the order: Solid Tissue, Spleen Cell, and Hematopoietic Stem Cell Transplantations. In contrast, the CD8 T cell response to H4 which represents ubiquitously expressed MiHAs decreases as the allogeneic hematopoietic cells increase. The hematopoieic cell-restricted distribution of H60 facilitates H60 dominance over H4. H60 is dominated by H4 in allogeneic skin transplantation.

mismatch of MiHA H4, a widely expressed MiHA, using the H4 congenic strain (B10.129-$H4^b$; Con-H4) as the donor (37). Collectively, these findings indicate that parenchymal cells do not naturally express H60; expression is restricted to hematopoietic cells and the H60-specific response is weak after solid tissue transplantations. In support of this, BALB.B→B6 cardiac engraftment and skin transplantation, neither of which features primary vascularization, H60 is subordinated to H4 (36, 39). However, when the BALB→ B6 heart transplantation involves a primary vascularization procedure, so that the BALB.B hematopoietic cells become more exposed to the B6 immune cells, H60 regains its dominance over H4 (H60>H4), as after BALB.B spleen cell immunization (30, 39). Similarly, H60 immunodominance is exaggerated during B6 anti-BALB.B GVH responses (7), when homeostatically proliferating B6 T cells are exposed to a large number of H60-positive host leukocytes. Thus, immunodominance of H60 is flexible and depends on the type of graft. H60 dominance is intensified by the abundance of H60-expressing allogeneic hematopoieic cells, due to its hematopoietic cell-restricted distribution (**Figure 1**).

Contribution From High Precursor Frequency in the Naïve T Cell Pool

Various factors affect antigen immunodominance and the immune hierarchy. Affinity for the MHC and the numbers of peptide/MHC complexes are crucial factors influencing immunodominance (40–42). However, the binding affinity of the cognate H60p LTFNYRNL to H-$2K^b$ is 3–10-fold lower than that of the $Ova_{257-264}$ SIINFEKL peptide, but is similar to that of the SGIVYIHL $H4^b$ CD8 epitope (Kd = 0.8 ± 0.05 nM), and empirical estimates of natural LTFNYRNL/H-$2K^b$ complexes (5–15 copies per cell) are not exceptionally high (15, 43). Thus, affinity and ligand density do not readily explain the unusually high immunodominance; H60-specific T cells expand at significant levels (to attain 7–8% of the peripheral peak) even in the presence of allo-MHC responses during the B6 anti-BALB/c (H-2^d) HVG response, and H60-targeted T cell cytotoxicity is detected at levels similar to that of H-2^d-targeted cytotoxicity during the haplo-MHC mismatched B6 anti-CB6F1 HVG response (31). H60 immunodominance is reproduced after immunization of B6 mice with the synthetic

H60 peptide; naïve protein expression is not involved (31). Rather, precursor cells are significantly frequent in the naïve CD8 T cell pool. The precursor frequencies (ca. 1/24,000–1/11,000 cells) are significantly higher than those of cells recognizing the H13 and HY subdominant MiHAs (such cells are in fact undetectable), and comparable to the level of cells reactive to a viral epitope VSV peptide (RGYVYQGL; ca. 1/49,000–1/18,000 cells) (31, 44). Additionally, the precursor TCR repertoire is diverse as revealed the high shannon entropy (average 5.8) and simpson index (0.99) of rearranged TCRβs sequences (45). Consequently, CD8 T cells with a wide spectrum of TCRs, thus almost all TCRVβs and the various CDR3s of each TCRVβ, are expanded in B6 mice immunized with spleen cells from Con-H60 mice (46). These features may explain why H60 is so immunogenic. However, because boosting the frequency of subdominant H13-reactive T cells via pre-immunization does not attenuate the dominance of H60 (30), not only the frequency and TCR diversity, but also the TCR avidity for H60p/H-2K^b of the precursors which are generated through selection processes in the B6 (H60^{null}) thymic environment likely play roles in establishing the immunodominance of H60 in the B6 T cell response.

DOES HEMATOPOIETIC CELL-RESTRICTED DISTRIBUTION AFFECT THYMIC SELECTION OF T CELLS SPECIFIC FOR H60?

As described above, the immunodominant H60 serves as useful model antigen when studying anti-MiHA allogeneic T cell response in MHC-matched allo-HSCT settings. Anti-H60 donor T cell responses can occur at two different levels. Mature donor T cells contained in the graft inoculum recognize host H60 and induce the GVL effect and GVHD. Also, donor-derived naïve T cells newly developed in the recipient thymus may recognize host H60 to induce the GVL effect and GVHD. Although many studies have focused on acute effects of the former T cells, *de novo* generated naïve T cells also can contribute to both the GVL effect and GVHD. Thus, it is important to understand thymic development of H60-specific naïve T cells. Below, we will describe natural thymic selection of H60-specific T cells and under allo-HSCT settings.

Incomplete Thymic Negative Selection of T Cells Specific for Self-Hematopoietic Cell-Restricted Antigens

It is well-established that T cells with specificity for ubiquitously expressed self-antigens are deleted in the thymus, preventing T cell-mediated autoimmunity (47). This is true for tissue-restricted antigens (TRAs) that are expressed only in certain peripheral tissues and cells; antigen-specific T cells for TRAs can be negatively selected due to AIRE-mediated promiscuous expression of certain genes (such as endocrine genes including insulin) by medullary thymic epithelial cells (mTECs) (48, 49). However, in recent years, it has become clear that some T cells escape thymic negative selection. In particular, certain TRA-specific T cells, especially those with low avidity TCRs, survive negative selection in the thymus and enter the periphery (50–53). Thymic negative selection against hematopoietic cell-restricted antigens (HRAs), especially natural HRAs such as H60, has not been studied in depth. Thymic dendritic cells (DCs) are known to be responsible for negative selection in the thymus (47, 54, 55). They have great capacity to delete thymocytes with high affinity/avidity TCRs for self-expressed antigens (direct presentation) and also those expressed by mTECs (cross-presentation) (56, 57). The conventional view has thus been that thymic deletion of T cells specific for HRA would be strict because of its thymic DC expression. However, our recent study using the natural antigen H60 as self- and allo-HRA revealed that some HRA-specific T cells survive thymic negative selection (45).

Our initial findings came from experiments using TCR-Tg mice of the B6 (H60^{null}) background strain, termed J15 mice, in which all T cells express TCRs originated from a high avidity anti-H60 clone (58). The J15 TCR has high specificity for H60, in that J15 T cells are strictly deleted in the thymus of Act-H60 Tg mice, but not in the thymus of Tg mice where a signal amino acid variant of H60 termed H60H (LTFHYRNL) is expressed under the control of the actin promoter (Act-H60H) (59, 60). Despite the high specificity and avidity, J15 T cells are incompletely deleted in the Con-H60 thymus even though the thymic DCs express H60. CD8 single positive (SP) thymocytes and splenic T cells were generated after crossing J15 and Con-H60 mice, although the numbers were 3–7-fold less and tetramer staining intensity was about 7-fold lower than those of B6 mice. Thus, J15 T cells expressing low avidity TCRs composed of transgenic TCRβ and endogenous TCRαs escaped negative selection in the Con-H60 thymus. Even under physiological conditions, H60-tetramer-binding polyclonal T cells can be detected among CD8 SP thymocytes and splenocytes of Con-H60 mice, albeit with lower tetramer-staining intensities and in numbers 2–2.5-fold lower than those of B6 mice. However, tetramer-binding cells are rarely detected in Act-H60 Tg mice. Thus, the incomplete deletion of T cells in the Con-H60 thymus is attributable to the natural hematopoietic cell-restricted expression pattern of H60. This finding allies with a study showing that limiting TRA expression to DCs results in incomplete deletion of thymic CD4 T cells (52).

Incomplete Thymic Negative Selection of T Cells Specific for Allo-HRA H60

In an H60-single antigen-mismatched allo-HSCT model, J15→Con-H60, donor-derived J15 T cells developing *de novo* are also only partially deleted in the thymus of Con-H60 recipients. Because radiation conditioning induces hematopoietic cell death in the recipient, negative selection may have not occurred. Thus, partial deletion in this setting was somewhat surprising. The mediators of such partial negative selection turned out to be radiation-resistant hematopoietic cells of the Con-H60 recipient: J15 T cells were positively selected in the J15→$\beta 2m^{-/-}$Con-H60 BMT, whereas partial deletion was preserved in the $\beta 2m^{-/-}$J15→Con-H60 BMT. Partial deletion of HRA-specific

CD8 T cells was also evident when Ova was expressed as an HRA in the recipient of OT-1 BM (OT-1→[Ova Tg→B6]) (45).

Therefore, deletion of T cells specific for HRAs may not be as strict as was conventionally thought. When limited numbers of DCs serve as the cognate thymic APC, the specific T cells are only partially deleted. Even when all thymic DCs are cognate APCs (as in Con-H60 mice), weak antigen-presentation by each APC (5–15 copies of the antigen-MHC complexes in the case of H60) may prevent strict deletion of the T cells. Thus, the hematopoietically-limited nature of H60 presentation allows low avidity T cells to escape thymic deletion.

A GVL EFFECT MEDIATED BY POST-THYMIC CD8 T CELL ESCAPEES SPECIFIC FOR HRA H60

In some animal models of allo-HSCT, hosts with chronic GVHD exhibit mTEC injuries (61–63). In recipients expressing Ova as a TRA, TRA-specific CD4 T cells are generated *de novo* because of mTEC damage occurring during the period of acute GVHD. Post-thymic T cells generated without negative selection trigger autoimmune-like disease in the context of a pro-inflammatory milieu (64). Thus, not just the failure of complete negative selection, but also acute GVHD-associated inflammation increases the likelihood that TRA-specific deletion escapees will generate autoimmune-like chronic GVHD.

In the case of H60, CD8 T cell escapees specific for HRA H60 consist of low-avidity T cells, exhibiting low tetramer-staining intensity. However, their TCR repertoire diversity is comparable to that of the B6 counterparts generated in the absence of negative selection. Escapees generated in B6→ Con-H60 BMT recipients are functional, producing IFN-γ and proliferating in response to H60 peptide-stimulation. More importantly, they have potent anti-leukemia effects. B6→ Con-H60 hosts showed tumor-free survival rates comparable to that of B6→ B6 hosts, because tumor cells were eliminated in an antigen-specific

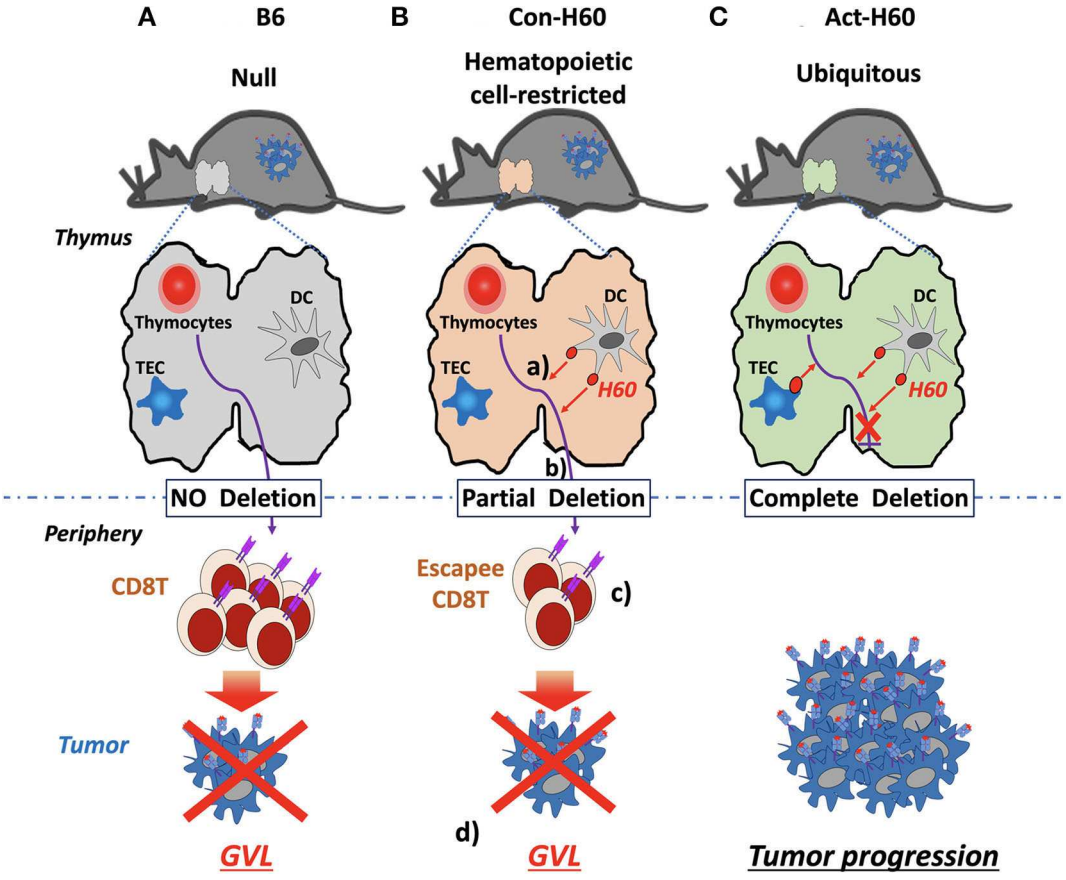

FIGURE 2 | GVL effects by H60 HRA-specific thymic deletion escapees in allo-HSCT. **(A)** In normal B6 recipients ($H60^{null}$), donor-derived T cells ($H60^{null}$ B6) specific for H60 are not deleted in the thymus because H60 expression is lacking. Surviving high-avidity H60-specific T cells mediate a GVL effect against H60-expressing hematologic tumors in the periphery. **(B)** In Con-H60 recipients (that express hematopoietic cell-restricted H60 on the B6 background): (a) donor-derived T cells specific for H60 are partially deleted by H60-expressing host dendritic cells (DCs) in the thymus; (b) low-avidity H60-specific T cells survive this negative selection; and (c) differentiate into effectors in the periphery. (d) These effectors mediate a GVL effect against H60-expressing hematologic tumors. **(C)** In Act-H60 recipients (that express H60 ubiquitously on the B6 background), donor-derived T cells specific for H60 are completely deleted by the H60-expressing thymic DCs and thymic epithelial cells (TEC). In the absence of surviving T cells specific for H60, H60-expressing hematologic tumors cannot be eliminated in an antigen-dependent manner.

manner (**Figures 2A,B**). Such GVL effects were not found for B6→Act-H60-Tg hosts because of the strict deletion of the H60-specific T cells (**Figure 2C**). Another critical point is that HRA H60-specific CD8 T cell escapees did not cause GVHD-like symptoms in B6→ Con-H60 hosts. However, when the numbers of escapees are non-physiologically high, as in the J15→ Con-H60 BMT, GVHD-like symptoms and mortality were observed in some hosts (<30%). Other findings have included donor leukocyte chimerism and expansion of H60-specific CD8 T cell escapees during the GVL response (45). Such data are clinically relevant as similar phenotypes are observed in leukemic patients showing favorable outcomes after allo-HSCT (12, 65, 66). Thus, hematopoietic cell-restricted MiHA-specific naïve T cells can develop in allo-HSCT hosts and contribute to the GVL effect with minimal GVHD, highlighting the potential utility of hematopoietic MiHA-mismatched HSCT in the clinic.

GVL EFFECTS MEDIATED BY DONOR MATURE CD8 T CELLS SPECIFIC FOR HRA H60

As we mentioned above, during the early phase of allo-HSCT, donor-derived mature T cells initiate acute GVH, and GVL responses. Donor-derived mature T cells specific for H60 expand greatly soon after transplantation, predominating the B6 anti-BALB.B GVH response (7, 67). However, depletion of T cells specific for H60 (and H4) within the graft prior to transplantation does not alleviate GVHD severity (68), indicating that donor-derived mature T cells specific for HRA H60 are not critical for inducing acute GVHD. It is thus clear that T cells responses raised against multiple MiHA-mismatch, rather than the just two MiHA, contribute to GVHD induction, consistent with the fact that an H4 single mismatch cannot induce acute GVHD (69). However, substantial GVL effects have been observed after transfer of T cells containing memory cells for H60 (70, 71). In MHC (H-2^b)-matched C3H.SW ($H60^{null}$)→ Con-H60 allo-BMT, transfer of CD8 T cells from H60-vaccinated donors, containing 2,600–5,000 H60-tetramer$^+$ cells, prolonged the survival of the $H60^+$ tumor-bearing hosts with chronic phase or blast crisis chronic myeloid leukemia (CML) (70, 71). The remarkable expansion of the H60-specific T cells (up to 56% of splenic CD8 T cells) and effective tumor killing, compared to the naïve T cell transfer, reflect inclusion in the transplant of central memory cells which can proliferate and differentiate into effector immediately upon antigen-restimulation. In addition to the direct cytotoxic effect, the ability to generate IFN-γ producing effectors immediately and in large numbers allows the memory T cell transfer to exert a powerful GVL effect: IFN-γ sensitizes GVL-resistant blast crisis CML and acute myeloid leukemia to T cell-mediated killing (71). Notably, this memory cell transfer strategy does not generate the GVL effects in hosts where H60 is ubiquitously expressed (70). The memory cells induce only mild hepatic GVHD, unlike the typical aggressive GVHD seen in hosts transferred with naïve T cells. Thus, memory T cells specific for an HRA may serve as tumor-targeting tools mediating a GVL effect. CD4 help is required for appropriate generation and expansion of memory CD8 T cells specific for cellular antigens including H60 (34, 35). Therefore, the development of strategies that include or supplement CD4 help factors will render amplification of memory T cells feasible. Collectively, GVL studies using H60 as a model HRA have validated the use of HRA-mismatched allo-HSCT and HRA-specific memory T cells to maximize GVL effects, while minimizing GVHD, in treatment of hematological malignancies.

CONCLUSION

We have reviewed the molecular characteristics of H60, a hematopoieic cell-restricted immunodominant MiHA, and the GVL effects of specific T cells. H60 allelism (*H60a*^{null} vs. *H60a*^{C}) and the hematopoietic cell-restricted distribution explain the mechanisms, such as the frequency, diversity, and avidity of reactive T cells, which underlie H60 immunodominance and the GVL effect of H60-specific T cells. Particularly, thymic deletion escapes of T cells with low avidity for HRA H60, and a GVL effect generated by the escapees against H60-positive tumor cells in the periphery have not been evaluated previously. These findings, together with the GVL effect generated by the transfer of memory T cells, emphasize the utility of HRA identification and the use of HRA-mismatched allo-HSCT to treat leukemia and lymphoma.

HRA-mismatched allo-HSCT, and the potential use of human HRAs such as HA-1 (*HMHA1*; VLH/RDDLLEA restricted by HLA-A*0201) and HA-2 (*MYO1G*; YIGEVLVSV/M restricted by HLA-A*0201) as targets for lysis of leukemic cells have been evaluated in clinic for years (72–75). The HA-1-mismatched allo-HSCT followed by HA-1-negative donor lymphocyte infusions successfully treated a relapse of HA-1^+ leukemia (12, 76, 77). T cell clones from patients with GVL in the absence of GVHD consistently did not react with non-hematopoietic cells, whereas those from GVHD patients were skewed to broadly expressed MiHAs (78). TCRs from high avidity HA-1-specific clones were used to develop memory T cells targeting leukemia (79). In this respect, identification of HRAs and their specific T cell clones is valuable. HRA expression may not be static, being possibly down-regulated by physiological, or pathological signals including IFNs (80, 81). However, MiHAs afford advantages compared to highly personalized tumor-associated neo-antigens, in that MiHAs shared by a group of people may allow germ-line based treatments. Moreover, the expression of certain HRAs, such as H60 and HA-1 (82–84), can be ectopically induced during carcinogenesis, extending the potential of HRA-based therapies to solid tumor target. Recently, a library of 39 novel MiHAs (restricted by HLA-A*02:01 or HLA-B*44:03) expressed on hematological cells has been reported (10). Molecular characterizations of these MiHAs and the reactive T cells will aid the utilization of HRAs as GVL targets. Our present review will assist in selection of HRAs for clinical applications.

In summary, mouse studies using H60 as a model HRA have yielded basic knowledge supporting the importance of strong

immunogenic HRAs, and donor-derived post-thymic T cells and memory T cells specific for such HRAs for generation of GVL effects. The $H60^{+}$ tumor targeting by post-thymic T cell was revealed using a single antigen-mismatched model. This will be extended to multi-antigen-mismatched allo-HSCT models. Also, future mechanistic studies on GVL mediation by H60-specific T cells will increase our ability to develop strategies that sustain anti-tumor effects while minimizing GVHD or autoimmune-like symptoms.

AUTHOR CONTRIBUTIONS

EC wrote the manuscript. KC provided critical insight and edited the manuscript. GN provided supportive experimental data. GN, WK, and MC designed the figures.

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MINI REVIEW
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The Connection Between Minor H Antigens and Neoantigens and the Missing Link in Their Prediction

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For hundreds of thousands of years, the human genome has extensively evolved, resulting in genetic variations in almost every gene. Immunological reflections of these genetic variations become clearly visible after an allogeneic stem cell transplantation (allo-SCT) as minor Histocompatibility (H) antigens. Minor H antigens are peptides cleaved from genetically encoded variable protein regions after which they are presented at the cell surface by HLA molecules. After allo-SCT with minor H antigen mismatches between donor and recipient, donor T cells recognize the minor H antigens of the recipient as foreign, evoking strong alloreactive immune responses. Studies in the late eighties have discovered that a subset of minor H antigens are encoded by hematopoietic system-specific genes. After allo-SCT, this subset is strictly expressed on the hematopoietic malignant cells and was therefore the first well-defined highly immunogenic group of tumor-specific antigens. In the last decade, neoantigens derived from genetic mutations in tumors have been identified as another group of immunogenic tumor-specific antigens. Therefore, hematopoietic minor H antigens and neoantigens are therapeutic equivalents. This review will connect our current knowledge about the immune biology and identification of minor H antigens and neoantigens leading to novel conclusions on their prediction.

Keywords: minor histocompatibility antigen, neoantigen, antigen prediction, antigen identification, reverse antigen identification strategy

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INTRODUCTION

Minor H Antigens: From Enigmatic to Well-Defined Transplantation-Antigens

Today, more than six decades after the first application of allogeneic stem cell transplantation (allo-SCT), scientists and clinicians are still impressed by the therapeutic Graft-versus-Tumor (GvT) effect established by donor T cells administered along with stem cells into the recipient (1). This therapeutic effect can be so powerful that patients can remain in long term remissions, even may be cured, after transplantation (2, 3). Therefore, allo-SCT is still being widely applied for several recurrent hematological malignancies, even though the therapeutic effects of allo-SCT are strongly associated with the development of life-threatening Graft-versus-Host-Disease (GvHD). The main mediators of GvHD as well as GvT are the alloreactive donor T cells directed at recipient antigens that are absent in the donor, responding to Major Histocompatibility Complex (MHC; HLA in

humans) molecules at the cell surface (4). However, GvT and especially GvHD still occur in about 40% of patients whose stem cell donors are completely HLA-identical, indicating the existence of an additional transplantation antigen system (5). These transplantation antigens were originally designated as minor Histocompatibility (H) antigens (6).

The nature of minor H antigens recognized by donor T cells remained an enigma for more than two decades. In the mid nineties, almost a decade after the identification of MHC-bound peptides as T cell epitopes (7) and the demonstration of structure and the peptide binding groove of MHC class I molecules (8, 9), pioneering studies conducted in mice and humans demonstrated that minor H antigens are polymorphic peptides presented by MHC molecules (10, 11). A subgroup of minor H antigens, the male-specific HY antigens, were derived from "male-specific" proteins encoded by genes located on the Y-chromosome (12). All other non-gender related minor H antigens identified to date are encoded by autosomal genes that have gained allelic polymorphism through evolution over thousands of years [reviewed in Oostvogels et al. (13)]. Although some analyses suggested that minor H antigens are mainly derived from oncological relevant genes (14), this idea was not embraced by all investigators. Any non-synonymous coding variation can give rise to an immunogenic minor H antigen after allo-SCT. Of these variations, single nucleotide polymorphisms (SNPs) leading to single amino acid substitutions are currently the most common for the generation of minor H antigens (10, 15–19). But also base-pair insertions, deletions (indels) or copy number variations (CNVs) contribute to the generation of polymorphic peptides that are recognized as minor H antigens at the cell surface (20).

The Concept of the Minor H Antigen-Targeted Immunotherapy

As soon as the molecular identity of minor H antigens was unraveled, it became clear why several minor H antigen-specific T cells isolated from transplanted patients lysed only hematopoietic cells, including hematopoietic tumor cells but not the cells derived from other tissues such as fibroblasts or keratinocytes (21). In all those cases the target minor H antigen was encoded by genes, which are solely expressed in the hematopoietic system (22). This discovery underlies the concept of minor H antigen-targeted immunotherapy, which aims at targeting hematopoiesis-specific minor H antigens, which would induce GvT without GvHD after allo-SCT. Also the newly developing minor H antigen negative donor-derived hematopoietic system would remain unharmed (23). The development of this concept fueled the efforts to identify new hematopoiesis-specific minor H antigens. To be broadly therapeutically applicable, such minor H antigens are ideally presented by common HLA-alleles and have a balanced population prevalence in order to get frequent minor H antigen disparities between donor and patient (24). Now, almost 25 years later, the research resulted in the identification of about 10 genuinely hematopoiesis-specific minor H antigens (18, 25–33), some of which have been or are being tested in early phase I/II clinical trials. The approaches used in these trials include treatment of allo-transplanted patients with *ex vivo* generated minor H antigen-specific T cells (34, 35), with T cell receptor (TCR)-gene transferred T cells (NCT03326921, ongoing) or vaccination of allo-transplanted patients with recipient- or donor-derived dendritic cells loaded with minor H antigen peptides (13, 36) or with minor H antigen encoding mRNA (NCT02528682, ongoing) (37). Nevertheless, except the HA-1, UTA2-1, and CD19 minor H antigens (25, 27, 29), all hematopoiesis-specific minor H antigens identified till now are either presented by infrequent HLA-alleles or display an unbalanced population frequency, which makes it highly challenging to enroll sufficient minor H antigen mismatched donor-patient pairs in clinical trials. Due to this issue, all current clinical translation attempts are either progressing very slowly (38) or even terminated due to poor accrual (NCT00943293). Thus, the efficient clinical translation of this highly personalized immunotherapy approach is still largely dependent on the development of solid strategies to identify clinically relevant hematopoiesis-specific minor H antigens. These efforts are relevant not only for the application of minor H antigen-targeted immunotherapy but also for immunotherapy aiming at targeting the so-called neoantigens, because the genetic, immunogenic, and therapeutic properties of hematopoietic minor H antigens and tumor-specific neoantigens display extreme similarities.

Similarities and Differences Between Hematopoietic Minor H Antigens and Tumor-Specific Neoantigens

Minor H antigens are the immunological reflections of evolutionary established genetic polymorphisms, while neoantigens are immunological reflections of tumor-specific genetic mutations (38, 39). Thus, from a genetic point of view, the only difference between these antigens is that minor H antigens are inherited, while neoantigens are not. For subsequent gene expression, antigen processing and HLA-mediated presentation, minor H antigens and neoantigens follow identical rules. These include that HLA class I (HLA-I) antigens are liberated from the polymorphic or mutated regions of intracellular proteins by (immuno)proteasomes in the cytosol, followed by ER-translocation via transporters associated with antigen processing (TAP) in order to be loaded into HLA-I molecules (40). Next to HLA-I restricted antigens that induce $CD8^{\pm}$ cytotoxic T cells, HLA class II (HLA-II) restricted antigens that induce $CD4^{\pm}$ T cells can also play important roles in anti-tumor responses. The proteolytic processing of HLA-II restricted minor H antigens and neoantigens is generally regulated by lysosomal enzymes, followed by HLA-DM assisted loading into the HLA-II peptide binding groove (41).

From the immunological point of view, the existence of minor H antigen- and neoantigen-specific T cells in the naïve T cell repertoire is likely similar. Both antigens are foreign to the immune system and therefore there is no negative selection for the high-affinity T cells reactive with minor H antigens and neoantigens in the thymus (42). Consequently, both antigens can induce very potent T cell immune responses. The basic difference

is that minor H antigens are solely immunogenic in a minor H antigen mismatched allo-SCT setting, while neoantigens can be readily immunogenic both in the allogeneic and autologous settings (38, 39).

Finally, from a clinical viewpoint, minor H antigens can be encoded by any polymorphic gene and are thus not tumor-specific antigens *per se*, as opposed to neoantigens. In fact, many minor H antigens are expressed by normal tissues and associate with the occurrence of detrimental GvHD as explained above (43). Nonetheless, this latter distinction does not apply for hematopoietic minor H antigens, which are tumor-specific antigens after an allo-SCT, similar to neoantigens (23, 24). This is because after allo-SCT the originally minor H antigen-positive normal hematopoietic system of the recipient is replaced by the minor H antigen-negative donor hematopoietic system. The only cells expressing the hematopoietic minor H antigens are the residual tumor cells. Therefore, it would not be wrong to state that hematopoietic minor H antigens in an allo-SCT setting are the equivalents of tumor-specific neoantigens. It should be noted that the replacement of residual minor H antigen positive host dendritic cells (DCs) after allo-SCT can take longer periods. These residual host DCs can therefore present endogenous hematopoietic minor H antigens to prime hematopoietic minor H antigen-specific T cells without the need for cross-presentation (44). In the case of neoantigens however, cross-presentation of the target antigen by DCs is an absolute requirement, because tumor cells are generally not able to prime T cells. Furthermore, specific targeting of either of these types of antigen is expected to exclusively generate a powerful anti-tumor effect without inducing direct damage to non-malignant cells.

From the therapeutic point of view, one final common and challenging aspect is the execution of clinical studies. As stated above, many minor H antigen-based clinical studies are facing with poor recruitment issues. Similar poor recruitment for adoptive T cell transfer trials is also expected for neoantigens due to the highly personalized character of most tumor-specific mutations. Since vaccination studies can include several antigens in one study, they are more easily applied (45) as compared to adoptive T cell transfer, but their success is still critically dependent on the development of effective (DC) vaccination strategies that can induce robust and long lasting T cell responses (46).

All these similarities between minor H antigens and neoantigens together show that it is of paramount importance to combine the knowledge of both fields toward the effective identification of both types of antigens and their application in the clinic.

Most Successful Methods for the Identification of Minor H Antigens and Neoantigens

In general, methods for the identification of a peptide antigen recognized by T cells fall into two main categories. The direct (forward) strategy aims to identify the antigen of a T cell clone that has already been isolated from a patient or a healthy individual. The "reverse" strategy follows the opposite direction through the isolation of a T cell clone that recognizes an *in silico* predicted antigen of interest (**Figures 1A,B**).

Forward Antigen Identification Strategies

There are several forward methods to identify minor H antigens and a fewer to identify neoantigens (**Figure 1A**). Initially, specialized biochemical peptide elution and fractionation techniques were used to identify peptides recognized by minor H antigen- and tumor-specific T cell clones (10, 12, 18, 25). At the same time, laborious cDNA library screening approaches were utilized to identify minor H antigen and tumor antigen encoding mRNA (47, 48). However, after the discovery that minor H antigens were encoded by inheritable genetic variations, genetic analyses were developed specifically for minor H antigens (32). Over the last 15 years, we and others have advanced these analyses from conventional pairwise linkage analysis into rapid and convenient SNP-based genome-wide association studies (15, 29, 49, 50). Moreover, we have implemented major resolution upgrades of those screens that initially used self-made databases toward publically available databases first from the HapMap Project and later from the 1,000 Genomes Project, which highly improved the success rate of genetic HLA-I and HLA-II restricted minor H antigen identification efforts (49). With these methods, a minor H antigen recognized by a T cell can be identified within 3–4 months (15). It is therefore not surprising that the vast majority of the more than 50 known minor H antigens to date has been identified by genetic linkage analyses (15, 27–29, 32, 33, 49, 51–53). Since neoantigens are not encoded in the germline and thus are not polymorphic in the population, the highly convenient forward genetic approaches are not applicable to their identification.

Despite its evident success, the forward T cell-to-antigen strategy has clear drawbacks when it comes to the identification of minor H antigens with a desired HLA-restriction, population frequency and tissue distribution. Although minor H antigen-specific T cell clones can be readily isolated from many, if not all, allo-transplanted patients, the available T cell isolation techniques cannot be adapted to isolate only those T cell clones with the required characteristics (24). All generated T cell clones need to be tested for the desired HLA restriction and minor H antigen frequency using cell line panels. Moreover, there are no convenient and reliable strategies to select T cell clones directed at minor H antigens expressed only in the hematopoietic system. A better and more convenient control of HLA restriction, population frequency and tissue distribution is key toward more efficient identification of clinically relevant minor H antigens.

The Reverse Antigen Identification Strategies

While forward methods hamper at the identification of clinically relevant minor H antigens and neoantigens, opportunities are offered by the "reverse immunology" approach. The reverse method first predicts potential T cell antigens based on *in silico* analyses of polymorphic or mutated genomic sites (**Figure 1B**). The 1,000 Genomes Project has cataloged most of human polymorphism and is therefore the database of choice for selection of putative minor H antigen encoding variations, preferably with a balanced allele frequency to allow

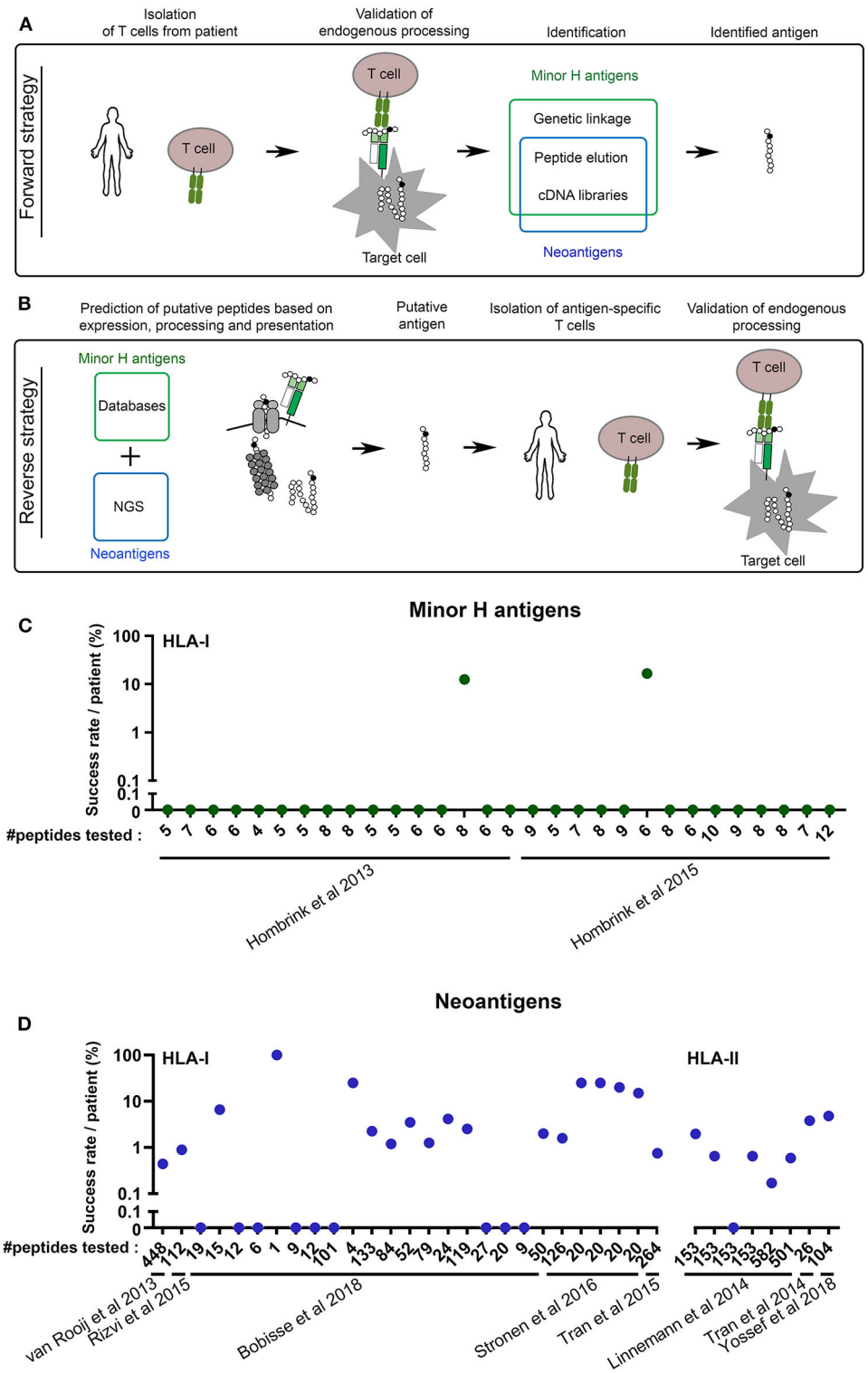

FIGURE 1 | The reverse antigen identification strategy still requires major improvement. **(A)** Schematic overview of the forward antigen identification strategy. **(B)** Schematic overview of the reverse antigen identification strategy. The cartoons depict processing by the proteasome, TAP transporter and HLA class I. NGS, Next Generation Sequencing. **(C,D)** The success rate of the reverse immunology approach is low. **(C)** From two papers in the field of minor H antigens, data were collected about the number of potential minor H antigens that were tested (#peptides tested) vs. the percentage of peptides against which T cell reactivity was detected or raised. Only papers were selected in which reactive T cells were confirmed to recognize the endogenous (or naturally processed) antigen. **(D)** The same was done with eight key papers in the field of neoantigens, five on HLA-I and three on HLA-II antigens.

for optimal donor-recipient disparity (54, 55). Because of the personalized character of neoantigens, the current state-of-the-art for mapping individual mutations is through tumor exome sequencing (56). Next tumor transcriptome analyses based on RNAseq or online databases are usually utilized to filter for tumor expression. Candidate minor H antigens should go through an additional selection for selective hematopoietic restricted expression. Finally, algorithms are applied to determine HLA-binding and sometimes the antigen processing efficiency for each possible peptide covering the polymorphism or mutation (56). The combined predictions for HLA-I presented peptides generally provide a score that accounts for the C-terminal cleavage of the protein by the proteasome, the TAP-mediated translocation of the peptide into the ER and the binding of the peptide to HLA with high "on-" and low "off-" rates. The multistep predictions are then validated by isolating the antigen-specific T cells from relevant patients or individuals. The last, but very essential steps are the confirmation that the targeted antigens are naturally processed and that these endogenous antigens are effectively recognized by the isolated T cells or their TCRs (**Figure 1B**).

The reverse strategy contained several highly challenging aspects until the last decade, which underlie the limited success of minor H antigen or neoantigen identification attempts in that period. Thanks to the recent advances in human genomics [e.g., RNAseq, exome sequencing, 1,000 Genomes Project (54, 56)], tissue expression profiling [e.g., Single Cell Expression Atlas, The Human Protein Atlas, BioGPS (57–59)], antigen processing and binding algorithms [e.g., NetCTL/IEDB (60, 61)] and large-scale peptide-specific T cell detection tools [e.g., UV-exchangeable HLA-I multimers, multimer barcoding (56)], the reverse methodology has majorly improved especially for HLA-I antigens.

This has led to the identification of a vast number of neoantigens, the antigen category for which the forward identification strategies offered only limited options. At the same time, the *in silico* predictions have even led to the generation of multiple libraries of thousands of putative new minor H antigens (55, 62). Nonetheless, these thousands of putative minor H antigens identified by such strategies have only resulted in the actual identification of a handful of minor H antigens (17, 26, 63), because to date none of these reverse strategies account for all minor H antigen-specific features (see below). The success of this gene-to-T cell approach highly depends on the strategy of antigen selection and intensity of T cell isolation efforts. In order to quantify the current efficiency of the reverse strategy, we have analyzed seven recent studies that applied a reverse strategy to identify novel minor H antigens or neoantigens (63–69). We only included studies from which we could extract the number of predicted antigens actually tested, as compared to the number of antigens that were endogenously expressed and against which a true T cell response could be raised. These analyses show that the efficiency of the reverse identification approach is between 0 and 20% (**Figures 1C,D**). These data argue that the reverse identification pipeline is currently far from optimal, with a dominant pool of false positive predicted candidates against which no T cell reactivity can be detected or raised (**Figures 1C,D**). One of the causes of suboptimal prediction is the incompleteness of the human reference proteome, despite huge progress in the last two decades. This is illustrated by the fact that many newly identified antigens were derived from supposedly non-coded regions (70–73). Furthermore, different efforts have shown that more stringent selection on expression and HLA binding score improves the success rate (66). Because not all neoantigens or minor H antigens behave according to these stringent criteria, we expect the number of false negative antigens to increase, which could effect the amount of therapeutic opportunities for individual patients in the long term. Thus, there is still much room for improvement of the reverse approach, which in theory is the best directed and straight forward strategy to identify HLA-I restricted hematopoiesis-specific minor H antigens as well as neoantigens.

For HLA-II restricted antigens, the development of a reverse method is even more complicated, because the rules for antigen processing and HLA-binding are more promiscuous and less defined. Nevertheless, some pioneering studies have combined minimalistic *in silico* analyses, without including antigen processing or HLA-binding predictions, but with large plasmid- or peptide-library screening strategies to identify HLA-II restricted neoantigen-specific T cell responses in cancer patients (67, 74, 75). Similar to HLA-I restricted antigens, these studies resulted in a low discovery rate (0–6%, **Figure 1D**). Recently, predictions for HLA-II binding have been incorporated in these analyses, but endogenous processing of immunogenic peptides was not confirmed (45, 76). So far, no HLA-II restricted minor H antigens have been identified following a reverse strategy. For a more successful identification of HLA-II restricted minor H antigens or neoantigens through reverse strategies, prediction algorithms for peptide processing and HLA-binding, but also cognate T cell detection tools (such as HLA-II multimers) still require revolutionary improvements.

As an additional layer of confirmation before isolating T cells, recent studies applied selection of candidate minor H antigen peptides from a large pool of HLA-I derived peptides as detected by mass spectrometry (**Figure 1C**) (63, 69, 77). Nonetheless, these studies also generated many false positive candidates, indicating that starting analyses from HLA-derived peptide repertoire may not necessarily compensate the current drawbacks of T cell epitope prediction algorithms.

Differential Peptide Processing and Presentation Is a Major Opportunity in Minor H Antigen and Neoantigen Reverse Identification

When studying the immunogenicity of genetic variations, it is not sufficient to consider only the antigen processing steps such as peptide cleavage, TAP translocation, HLA binding. The proper execution of these processing steps is definitely required, but not sufficient for the majority of minor H antigens and neoantigens to become immunogenic. This is because the immunogenicity of a polymorphic or mutated peptide depends on the existence of peptide-specific T cells in the (donor) T cell repertoire. The extent of the T cell repertoire against a specific antigen can be negatively affected by the presence of similar antigens in the HLA-presented peptidome during thymic development (78).

TABLE 1 | The majority of minor H antigens (30/50) identified by an unbiased forward strategy are (predicted to be) differentially processed.

Levels of differential peptide processing	Minor H antigen	HLA restriction[a]	Peptide sequence[b,c]	C-terminal cleavage score[c,d]	TAP score[c,d]	HLA affinity score (nM)[c,d]	References
Transcription	UGT2B17/A29	HLA-A*29:02	**AELLNIPFLY**				(79)
			–				
	UGT2B17/B44	HLA-B*44:03	**AELLNIPFLY**				(79)
			–				
	UGT2B17/A2	HLA-A*02:06	**CVATMIFMI**				(50)
			–				
	ACC-6	HLA-B*44:02/03	**MEIFIEVFSHF**				(31)
			–				
	ZAPHIR	HLA-B*07:02	**IPRDSWWVEL**				(80)
			–				
Translation	LRH-1	HLA-B*07:02	**TPNQRQNVC**				(28)
			–				
	PANE1	HLA-A*03:01	**RVWDLPGVLK**				(81)
			–				
C-terminal or internal proteasome cleavage	ACC-4	HLA-A*31:01	ATLPLLCAR	**0.26**	0.68	17	(82)
			ATLPLLCAG	**0.03**	−0.57	11313	
	ACC-5	HLA-A*33:03	WATLPLLCAR	**0.26**	0.65	210	(82)
			WATLPLLCAG	**0.03**	−0.59	29173	
	HA-3[e]	HLA-A*01:01	VTEPGTAQY	**0.97**	1.25	13	(16)
			VMEPGTAQY	**0.97**	1.31	134	
	SP110[e]	HLA-A*03:01	SLPRGTSTPK			54	(83)
			SLPGGTSTPK			155	
	LB-FUCA2-1V	HLA-B*0702	RLRQVGSWL	**0.90**	0.48	38	(84)
			RLRQMGSWL	**0.53**	0.48	24	
	LB-GEMIN4-1V	HLA-B*07:02	FPALRFVEV	**0.97**	0.04	65	(53)
			FPALRFVEE	**0.24**	−0.78	3208	
	LB-GEMIN4-2V	HLA-B*08:01	FPALRFVEV	**0.97**	0.04	23	(71)
			FPALRFVEE	**0.24**	−0.78	405	
TAP transport	HA-8	HLA-A*02:01	RTLDKVLEV	0.96	**0.23**	35	(30)
			PTLDKVLEV	0.96	**−0.08**	3665	
HLA-binding	HA-1	HLA-A*02:01	VLHDDLLEA	0.95	−0.19	**29**	(25)
			VLRDDLLEA	0.93	−0.18	**321**	
	HA-2	HLA-A*02:01	YIGEVLVSV	0.96	0.12	**7**	(10)
			YIGEVLVSM	0.96	0.11	**58**	
	TRIM22	HLA-A*02:01	MAVPPCCIGV	0.95	0.17	**620**	(85)
			MAVPPCRIGV	0.89	0.17	**3046**	
	LB-APOBEC3B-1K	HLA-B*07:02	KPQYHAEMCF	0.26	0.95	**278**	(53)
			EPQYHAEMCF	0.26	0.82	**9507**	
	LB-BCAT2-1R	HLA-B*07:02	QPRRALLFVIL	0.94	0.33	**253**	(53)
			QPTRALLFVIL	0.92	0.30	**2753**	
	DPH1	HLA-B*57:01	SVLPEVDVW	0.45	0.50	**217**	(34)
			SLLPEVDVW	0.59	0.44	**1582**	

(Continued)

TABLE 1 | Continued

Levels of differential peptide processing	Minor H antigen	HLA restriction[a]	Peptide sequence[b,c]	C-terminal cleavage score[c,d]	TAP score[c,d]	MHC affinity score (nM)[c,d]	References
	LB-TRIP10-1EPC	HLA-B*40:01	GEPQDLCTL	0.96	0.26	**176**	(52)
			GGSQDLGTL	0.87	0.21	**15676**	
	LB-C16ORF-1R	HLA-B*07:02	RPCPSVGLSFL	0.9	0.38	**643**	(71)
			WPCPSVGLSFL	0.9	0.26	**3010**	
	LB-NCAPD3-1Q	HLA-A*02:01	WLQGVVPVV	0.91	0.18	**13**	(71)
			WLRGVVPVV	0.91	0.22	**108**	
	UTA2-1	HLA-A*02:01	QLLNSVLTL	0.97	0.46	**39**	(27)
			QLPNSVLTL	0.97	0.45	**222**	
	LB-TMEM8A-1I	HLA-B*07:02	RPRSVTIQPLL	0.97	0.41	**11**	(71)
			RPRSVTVQPLL	0.97	0.41	**28826**	
	LB-ERAP1-1R	HLA-B*07:02	HPRQEQIALLA	0.96	−0.45	**692**	(53)
			HPPQEQIALLA	0.97	−0.48	**12460**	
	LB-ADIR-1F	HLA-A*02:01	SVAPALALSPA	0.89	−0.08	**490**	(86)
			SVAPALALFPA	0.91	−0.08	**1555**	
TCR affinity	HB-1	HLA-B*44:03	**EEKRGSLHVW**	0.9	0.29	184	(48)
			EEKRGSLYVW	0.89	0.29	188	
	ACC1	HLA-A*24:02	**DYLQYVLQI**	0.9	0.20	115	(31)
			DYLQCVLQI	0.77	0.20	197	
Yet unknown	LB-ECGF-1	HLA-B*07:02	RPHAIRRPLAL	0.91	0.42	9	(73)
			RPRAIRRPLAL	0.91	0.43	5	
	SLC5A1	HLA-B*40:02	AEATANGGLAL	0.96	0.49	48	(50)
			AEPTANGGLAL	0.96	0.48	50	
	LB-WNK1-1I	HLA-A*02:01	RTLSPEIITV	0.97	0.30	58	(53)
			RTLSPEMITV	0.95	0.30	78	
	LB-NDC80-1P	HLA-A*02:01	HLEEQIPKV	0.97	0.09	82	(71)
			HLEEQIAKV	0.97	0.09	184	
	LB-ZDHHC6-1Y	HLA-B*07:02	RPRYWILLVKI	0.97	0.23	338	(71)
			RPRHWILLVKI	0.95	0.18	334	
	LB-SON-1R	HLA-B*40:01	SETKQRTVL	0.92	0.37	58	(52)
			SETKQCTVL	0.95	0.37	28	
	LB-SWAP70-1Q	HLA-B*40:01	MEQLEQLEL	0.94	0.43	178	(52)
			MEQLEELEL	0.92	0.43	141	
	LB-NUP133-1R	HLA-B*40:01	SEDLILCRL	0.90	0.28	194	(52)
			SEDLILCQL	0.90	0.28	80	
	P2RX7	HLA-A*29:02	WFHHCHPKY	0.95	1.40	6	(34)
			WFHHCRPKY	0.85	1.40	15	
	LB-TTK-1D	HLA-A*02:01	RLHDGRVFV	0.89	0.26	30	(51)
			RLHEGRVFV	0.84	0.26	33	
	LB-EBI3-1I	HLA-B*07:02	RPRARYYIQV	0.96	0.15	26	(53)
			RPRARYYVQV	0.96	0.15	19	
	LB-ARHGDIB-1R	HLA-B*07:02	LPRACWREA	0.42	−0.07	10	(53)
			LPRACWPEA	0.42	−0.07	35	
	LB-SSR1-1S	HLA-A*02:01	VLFRGGPRGSLAVA	0.89	−0.14	1403	(87)
			VLFRGGPRGLLAVA	0.86	−0.14	649	
	LB-PRCP-1D	HLA-A*02:01	FMWDVAEDL	0.92	0.49	8	(53)
			FMWDVAEEL	0.95	0.49	3	

(Continued)

TABLE 1 | Continued

Levels of differential peptide processing	Minor H antigen	HLA restriction[a]	Peptide sequence[b,c]	C-terminal cleavage score[c,d]	TAP score[c,d]	MHC affinity score (nM)[c,d]	References
	LB-MOB3A-1C	HLA-B*07:02	CPRPGTWTC	NA[f]	−0.13	442	(71)
			SPRPGTWTC		−0.09	69	
	LB-PNP-1S	HLA-B*13:01	TQAQIFDYSEI	0.57	0.28	NA[g]	(71)
			TQAQIFDYGEI	0.4	0.28		
	LB-GSTP1-1V	HLA-B*08:01	DLRCKYVSL	0.77	0.24	8	(71)
			DLRCKYISL	0.71	0.24	13	
	C19ORF48	HLA-A*02:01	TAWPGAPEV	0.97	0.38	163	(72)
			TAWPGAPGV	0.96	0.38	268	
Predicted not to bind HLA[h]	LB-C19ORF48-2E	HLA-B*51:01	TAWPGAPEV	0.97	0.38	24822	(71)
			TAWPGAPGV	0.96	0.38	25920	
	LB-PDCD11-1F	HLA-B*07:02	GPDSSKTFLCL	0.97	0.15	7364	(53)
			GPDSSKTLLCL	0.96	0.15	7408	
	LB-ZNFX1-1Q	HLA-B*40:01	NEIEDVWQLDL	0.94	0.47	5834	(71)
			NEIEDVWHLDL	0.96	0.47	4026	
	LB-APOBEC3B-1K	HLA-B*08:01	KPQYHAEMCF	0.26	0.95	9287	(71)
			EPQYHAEMCF	0.26	0.82	4709	
	LB-CCL4-1T	HLA-A*02:01	CADPSETWV	0.15	0.08	8014	(71)
			CADPSESWV	0.29	0.08	9995	

[a] *For B*44:02/03 binding mHags, only scores for B*44:03 are depicted.*

[b] *The upper peptide sequence corresponds to minor H antigen and the bottom to allelic counterpart.*

[c] *Bold values indicate the level of (predicted) differential peptide processing.*

[d] *Predictions made by NetChop3.1, IEDB and NetMHC4.0.*

[e] *HA-3 and SP110 are generated through differential internal proteasome cleavage and proteosome-catalyzed peptide splicing.*

[f] *The C-terminus of the MOB3A antigen is the C-terminus of the MOB3A protein.*

[g] *The HLA-B*13:01 binding prediction is not available at NetMHC4.0, but both peptides harbor the HLA-B*13 binding motif.*

[h] *Because the NetMHC4.0 affinity scores indicated no binding to HLA (>> 1,000 nM), it is currently complicated to assess potential differential HLA-binding, so we excluded these five minor H antigens from the analyses.*

Since in most cases minor H antigens and neoantigens differ only in a single amino acid from their respective allelic or wildtype counterpart peptides, it is crucial to consider potential effects on the shaping of the T cell repertoire.

If both mutated and wild type peptides are equally well-presented at the cell surface, then T cells may discriminate between these two peptides depending on the position of the amino acid substitution. This is for instance the case for two minor H antigens that both have a single amino acid substitution due to a SNP, HB-1 and ACC1 (**Table 1**) (26, 32). Separate T cell clones have been isolated that specifically recognize either one or the other allelic peptide at the cell surface (26, 32).

However, if the amino acid alteration is not at a position in the peptide that is exposed to the TCR, and if it is not affecting peptide conformation, then T cells can not discriminate between two of such slightly different peptides. In this case, the allelic or wildtype peptide variants already induce the cognate T cells to be deleted from the T cell repertoire by negative selection in the thymus, similar to self-peptide-reactive T cells (42). Consequently, individuals (donors or patients) lack the majority of the minor H antigen or neoantigen-specific T cells before even being exposed to these antigens. Furthermore, the few T cells in the repertoire that are antigen-specific will have a low affinity TCR with cross-reactivity against the allelic or wildtype peptide. Therapeutic use of such T cells may put patients at risk of detrimental self-recognition on healthy cells, similar to what has been observed for tumor antigens MART-1 and MAGE-A3 (88–91).

Such restrictions do not apply for peptide pairs that are differentially expressed on the cell surface. In fact, for several minor H antigens this is the case. For instance HA-1-, HA-2- and HA-3-specific T cells make no distinction between the allelic peptides (16–18, 25). The strong immunogenicity of such minor H antigens occurs due to the fact that the non-immunogenic peptide has impaired HLA-binding (HA-1) (92), proteasome cleavage (HA-3) (16), TAP translocation (HA-8) (30) or even a yet unknown processing event (HA-2) (**Table 1**). Similar one-sided lack of peptide presentation occurs if the genetic variation causes loss of conventional gene transcription such as through alternative splicing (31) or loss of genomic DNA (**Table 1**) (79). Finally, alternative translation events may also cause differential surface expression, for example due to a frame shift (28) or the introduction of a stop-codon (81) (**Table 1**). Moreover, *in silico* analysis of all currently known HLA-I restricted minor H antigens, which have been identified using unbiased forward strategies, revealed that at least 28/48 of the non-immunogenic counterpart peptides are likely not expressed on the cell surface. This is probably an underestimation because various parameters that

affect intracellular peptide processing were (largely) disregarded in this overview, such as proteosome-catalyzed peptide splicing, internal proteosomal cleavage, ERAP1 trimming (16, 83, 93). In addition, the prediction algorithms have been trained on positive datasets and therefore currently have limited power to predict the absence of processing. Taken together, this dataset suggests that more than 58% of genetically variable antigens are immunogenic because the non-immunogenic peptide is simply not present on the cell surface (**Table 1**). Mono-allelic (by some investigators called as "dominant") presentation was also seen in mass spectrometry data of HLA-I derived peptides (77, 94).

Recently, SNP-induced differential processing was also found for an HLA-II restricted minor H antigen (95), indicating that development of HLA-II epitope processing prediction algorithms may be valuable for future identification of immunogenic HLA-II presented antigens.

Strikingly, in the exploding field of neoantigen prediction models, differential surface expression of mutated vs. wild type peptide is largely neglected, which might be a reason of the large number of false positive neoantigen predictions (**Figure 1D**). Thus, the addition of a specific differential surface presentation module, based on the molecular features of minor H antigens and their allelic counterparts, to the current prediction models may improve both minor H antigen and neoantigen reverse identification strategies.

CONCLUSIONS AND FUTURE DIRECTIONS

There is no doubt that the genetic alterations encoding hematopoietic minor H antigens in the allo-SCT setting and neoantigens in the autologous setting can induce potent T cell responses in patients. For clinical application of both antigen types, the most important challenge is to include sufficient patients in the clinical trials. It is currently unclear what the best strategy will be to provide the personalized immunotherapy necessary to target either of these antigens. Furthermore, there are also major challenges for their identification. The current T cell epitope prediction algorithms need significant improvement. We here postulate based on the published data of the last 25 years that also predictions for the non-immunogenic allelic or wild type peptide should be included in the algorithms. Selecting only those candidate peptides that will be differentially expressed at the cell surface may increase the success rate to detect or raise antigen-specific T cells from the naïve repertoire.

AUTHOR CONTRIBUTIONS

TM and RS developed the concept, designed, and wrote the manuscript. AX performed literature analyses and generated the figures.

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HA-1H T-Cell Receptor Gene Transfer to Redirect Virus-Specific T Cells for Treatment of Hematological Malignancies After Allogeneic Stem Cell Transplantation: A Phase 1 Clinical Study

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Graft-vs.-leukemia (GVL) reactivity after HLA-matched allogeneic stem cell transplantation (alloSCT) is mainly mediated by donor T cells recognizing minor histocompatibility antigens (MiHA). If MiHA are targeted that are exclusively expressed on hematopoietic cells of recipient origin, selective GVL reactivity without severe graft-vs.-host-disease (GVHD) may occur. In this phase I study we explored HA-1H TCR gene transfer into T cells harvested from the HA-1H negative stem-cell donor to treat HA-1H positive HLA-A*02:01 positive patients with high-risk leukemia after alloSCT. HA-1H is a hematopoiesis-restricted MiHA presented in HLA-A*02:01. Since we previously demonstrated that donor-derived virus-specific T-cell infusions did not result in GVHD, we used donor-derived EBV and/or CMV-specific T-cells to be redirected by HA-1H TCR. EBV and/or CMV-specific T-cells were purified, retrovirally transduced with HA-1H TCR, and expanded. Validation experiments illustrated dual recognition of viral antigens and HA-1H by HA-1H TCR-engineered virus-specific T-cells. Release criteria included products containing more than 60% antigen-specific T-cells. Patients with high risk leukemia following T-cell depleted alloSCT in complete or partial remission were eligible. HA-1H TCR T-cells were infused 8 and 14 weeks after alloSCT without additional pre-conditioning chemotherapy. For 4/9 included patients no appropriate products could be made. Their donors were all CMV-negative, thereby restricting the production process to EBV-specific T-cells. For 5 patients a total of 10 products could be made meeting the release criteria containing 3–280 $\times$ 10^6 virus and/or HA-1H TCR T-cells. No infusion-related toxicity, delayed toxicity or GVHD occurred. One patient with relapsed AML at time of infusions died due to rapidly progressing disease. Four patients were in remission at time of infusion. Two patients died of infections during follow-up, not

likely related to the infusion. Two patients are alive and well without GVHD. In 2 patients persistence of HA-1H TCR T-cells could be illustrated correlating with viral reactivation, but no overt *in-vivo* expansion of infused T-cells was observed. In conclusion, HA-1H TCR-redirected virus-specific T-cells could be made and safely infused in 5 patients with high-risk AML, but overall feasibility and efficacy was too low to warrant further clinical development using this strategy. New strategies will be explored using patient-derived donor T-cells isolated after transplantation transduced with HA-1H-specific TCR to be infused following immune conditioning.

Keywords: HA-1, TCR gene transfer, minor histocompatibility antigen, allogeneic stem cell transplantation, graft-vs.-tumor effect

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (alloSCT) is used to induce or consolidate long-term remissions in patients with hematological malignancies. Although chemotherapy and/or irradiation is part of the essential conditioning treatment to allow engraftment of donor hematopoietic cells, the advantage of alloSCT over high dose chemotherapy or autologous stem cell transplantation is the potential profound effect of the alloimmune response mediated by donor T cells resulting in eradication or persistent control of the malignant hematopoietic clone in the patient (1). After HLA-matched alloSCT, this graft vs. leukemia (GVL) reactivity is mainly mediated by donor T cells recognizing minor histocompatibility antigens (MiHA) on recipient hematopoietic cells (2–6). If donor T cells recognize MiHA expressed on both recipient hematopoietic and non-hematopoietic cells, GVL reactivity is frequently accompanied by graft-vs.-host disease (GVHD) (7–10). If MiHA are targeted that are exclusively expressed on hematopoietic cells of recipient origin, selective GVL reactivity will occur coinciding with conversion to full donor chimerism of the hematopoietic system (11). Several MiHA have been reported to be selectively expressed on hematopoietic cells, and may therefore be targets for a specific GVL reactivity (12, 13). Although various factors may influence the balance between GVL and GVHD reactivity including dosing and timing of donor T-cell infusion, predictable selective induction of GVL reactivity appears to require infusion or induction of donor T cells that specifically target antigens that are selectively expressed on (malignant) hematopoietic cells of the patient (1, 14–17).

HA-1H is a MiHA with a population frequency of 30% which is selectively presented by the restriction allele HLA-A*02:01 on cells from hematopoietic origin, which can be recognized by T cells from HLA-A*02:01 positive individuals homozygous for the allelic counterpart HA-1R, lacking HA-1H (18, 19). We have illustrated that HA-1H-specific T cells can be found in the majority of homozygous HA-1R HLA-A*02:01 positive donors (20). We have demonstrated that donor T cells recognizing HA-1H can contribute to a specific GVL reactivity in the absence of severe GVHD (21). However, direct purification of HA-1H-specific T cells from donor peripheral blood to be used as therapeutic reagent has been difficult to achieve due to low frequencies. We therefore previously attempted to expand HA-1H specific T-cell lines using an *in-vitro* culture protocol. Although we have demonstrated that HA-1H-specific T-cell lines could be generated and infused into patients without toxicity, *in-vivo* expansion and clinical benefit could not be illustrated (20).

T-cell receptor (TCR) gene transfer appears to be an attractive *in-vitro* strategy to generate large numbers of antigen specific T cells that can be used for adoptive transfer. Autologous T cells modified to induce a TCR targeting an antigen of choice have been demonstrated to have clinical effectiveness after transfer into patients with solid tumors (22–25). Based on these encouraging results, we hypothesized that donor T cells engineered to express an HA-1H-specific TCR may be used to eliminate patient hematopoiesis including the malignant clone in HA-1H positive patients transplanted with an HA-1H negative (homozygous HA-1R positive) donor. Since unselected donor T cells may induce GVHD when infused into patients after alloSCT, we hypothesized that engineering virus-specific T cells from donor origin to express the HA-1H TCR would create a therapeutic product unlikely to induce GVHD. We and others have illustrated that the infusion of virus-specific T cells from donor origin into patients after alloSCT can have a profound anti-viral reactivity without toxicity (26–32). In addition, virus-specific T cells engineered to coexpress tumor-specific receptors demonstrated improved persistence after treatment of individuals with neuroblastoma (33). Therefore, T cells harboring both the endogenous virus-specific TCR and the transferred HA-1H TCR may have both beneficial specificities. To ensure appropriate expression of the HA-1H TCR in the virus-specific T cells and limit the risk of miss-paired dimerization between the endogenous and exogenous TCR, we used a codon optimized cysteine modified TCR, in which the TCR-α and -β chains were linked by a T2A sequence (34). The good manufacturing practice (GMP) grade production of HA-1H TCR transduced virus-specific cells for this HA-1H TCR gene therapy study was established by using MHC-I-Streptamer-based isolation technology and subsequent transduction with the HA-1H TCR using retroviral vectors (35).

In this phase I clinical study we explored the feasibility to generate HA-1H TCR gene transduced CMV or EBV-specific T cells harvested from the stem cell donor to obtain larger numbers of HA-1H-specific T cells and treat HLA-A*02:01 positive HA-1H positive patients with hematological malignancies, and evaluated

TABLE 1 | Characteristics of starting material and release specifications of generated products.

Donor	EBV status donor	CMV status donor	Line	Streptamer 1	Streptamer 2	Starting material MNC (10^6)	Starting material Strept 1 (%)	Starting material Strept 2 (%)	Directly after isolation Cells (10^6)	Directly after isolation Strept 1 (%)	Directly after isolation Strept 2 (%)	End of culture Cells (10^6)	End of culture CD3 (%)	End of culture Strept 1 (%)	End of culture Strept 2 (%)	End of culture HA1-TCR (%)	End of culture Total tetramer pos (%)
001	pos	pos	1	CMV-pp50-A1-VTE	EBV-BZLF1-B8-RAK	1590	1.2	1.2	10.5	49	45	283	99	27	33	39	94
			2	CMV-pp50-A1-VTE	EBV-BZLF1-B8-RAK	1890	1.2	1.8	8.3	52	45	228	98	27	26	36	97
002	pos	neg	1	EBV-BMLF1-A2-GLC	–	2351	0.2	–	1.7	72	–	3.1	99	59	–	41	100
			2	EBV-BMLF1-A2-GLC	–	2341	0.2	–	0.6	n.d.	–	2.9	97	44	–	30	83
003	pos	neg	1	EBV-BMLF1-A2-GLC	EBV-BZLF1-B8-RAK	2272	0.05	0.53	7.6	2.6	49	101	97	13	30	30	74
			2	EBV-BMLF1-A2-GLC	EBV-BZLF1-B8-RAK	2024	0.04	0.55	3.8	2.8	52	15	97	15	32	18	78
004	pos	neg	1*														
005	pos	pos	1	CMV-pp65-A2-NLV	EBV-EBNA3A-B7-RPP	1480	0.04	0.07	0.8	17	36	88	99	4.5	56	35	97
			2	CMV-pp65-A2-NLV	EBV-EBNA3A-B7-RPP	985	0.03	0.05	0.22	n.d.	n.d.	54	96	3.6	58	33	98
006	pos	neg	1*														
007	pos	neg	1	EBV-EBNA3A-B7-RPP	EBV-BZLF1-B8-RAK	1826	0.1	1.5	10.4	8	84	44	99	58	9.3	11	97
			2*	EBV-EBNA3A-B7-RPP	EBV-BZLF1-B8-RAK	2000	0.1	1.4	4.6	10	87	21.6	99	64	15	13	99
008	pos	neg	1*														
009	pos	neg	1*	EBV-BMLF1-A2-GLC	–	2074	0.1	–	0.7	80	–	0.16	84	72	–	5	77

*No infusion of HA1H TCR-transduced T cells.

potential toxicity and efficacy. After prophylactic infusion of HA-1H TCR-transduced CMV or EBV-specific T cells 8 and 14 weeks after T-cell depleted alloSCT with prescheduled postponed donor lymphocyte infusion (DLI) 6 months after alloSCT (17, 36), no infusion-associated toxicity, delayed toxicity, or GVHD was observed. In addition, persistence or expansion of HA-1H TCR transduced T cells was observed in 3 out of 5 patients. However, overall feasibility and efficacy was too low to allow further development of this specific therapeutic product. New strategies will be explored to evaluate potential efficacy of HA-1H TCR T-cells to control recurrence of hematological malignancies of HLA-A*02:01 positive HA-1H positive patients transplanted with an HA-1H negative donor.

MATERIALS AND METHODS

Generation of HA-1H TCR-Transduced CMV or EBV-Specific T Cells

HA-1H TCR-transduced CMV and/or EBV-specific T-cell products were generated as described previously (35). In short, either one or two virus-specific T-cell populations were isolated from a donor leukapheresis product harvested prior to the G-CSF mobilization and cryopreserved until further use. Virus-specific T-cell populations were isolated using the MHC-I-*Strep*tamer isolation technology (Juno Therapeutics GmbH, a Celgene company (formerly Stage Therapeutics), Munich, Germany). Isolation complexes (MHC-I-*Strep*tamers) were generated per T-cell specificity by incubation of peptide-loaded MHC-I-*Strep*-tag fusion proteins with magnetically labeled *Strep*-Tactin (*Strep*-Tactin nanobeads). The pool of MHC-I-*Strep*tamers was incubated with 1–2 × 10^9 donor PBMC for 45 min at 4°C. MHC-I-*Strep*tamer-bound cells were isolated using a CliniMACS Plus instrument (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. MHC-I-*Strep*tamers were dissociated from the positively isolated cells using 1 mM of D-Biotin. Selected virus-specific T cells were cultured with irradiated (25 Gy) autologous peripheral blood mononuclear cells (PBMNC) as feeder cells (ratio 1:5) in T-cell culture medium consisting of Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Basel, Switzerland) supplemented with 10% human serum, 100 IU/mL IL-2 (Proleukin®/Aldesleukin, Novartis, Arnhem, Netherlands) and 10 ng/mL interleukin-15 (Cellgenix, Freiburg, Germany). The virus-specific T cells were transduced 2–3 days after isolation with GMP-grade retroviral supernatant encoding the HA-1H TCR (EUFETS GmbH, Idar Oberstein, Germany) spun down on retronectin-coated (15 μg/well; Takara Bio, St-Germain-en-Laye, France) 24-wells clear flat-bottomed microplates (Greiner Bio-one, Alphen aan den Rijn, Netherlands) for at least 4 h at 37°C. The cells were subsequently cultured for 10–14 days in T-cell culture medium.

Study Design

The study *Administration of HA-1H TCR-transduced virus-specific T cells after allogeneic stem cell transplantation in patients with high risk leukemia* was registered at www.clinicaltrialsregister.eu as EudraCT number 2010-024625-20.

TABLE 2 | Characteristics of patients who received HA-1H TCR-transduced T cells.

Study ID	001	002	003	005	007
Age	51	36	65	47	51
Gender	Female	Female	Female	Female	Male
Disease	Therapy related AML	AML	AML	AML	B-LBL
Cytogenetics/molecular diagnostics	t (9,11) and t (1,15)	NCA	NPM1+ FLT3+	Monosomal karyotype	MLL+ t (4,11)
Number of infusions	2	2	2	2	1
Transplant manipulation	CD34 selection	Alemtuzumab	Alemtuzumab	Alemtuzumab	Alemtuzumab
Stem cell donor	MUD	Sibling	MUD	Sibling	MUD
Conditioning regimen	MA	NMA	NMA	MA	MA
Patient chimerism at first infusion (MNC-leucocytes-granulocytes)	0-0-0	1-1-?	0-0-0	0-0-0	0-0-0
Patient chimerism at second infusion (MNC-leucocytes-granulocytes)	0-0-0	1-1-2	0-0-0	83-77-21	
CMV load in serum at first infusion	3.2	0	0	2.5	0
CMV load in serum at second infusion	2.3	0	0	0	
Highest detectable CMV load in serum (weeks after first infusion)	4.5 (11)	0	0	2.4 (3)	0
EBV load in serum at first infusion	0	0	0	0	0
EBV load in serum at second infusion	0	0	0	0	
Highest detectable EBV load in serum (weeks after first infusion)	0	0	0	0	5.7 (7)
Development of GVHD (weeks after first infusion)	No	No	No	No	No
Infusion of standard care DLI 6 months after alloSCT	No	Yes	Yes	No	No
Adverse events (between first infusion and 6 months post alloSCT)	• Pulmonal aspergillus • Candidaemia • Parvovirus • Bacteriemia S. Haemolyticus	None	None	Relapse AML	• PTLD • Pulmonal aspergillus
Duration of follow up in weeks after first infusion	19	234	224	19	7
Alive at last follow up	No	Yes	Yes	No	No
Cause of death	Multiple infections			Relapse AML	PTLD

AML, acute myeloid leukemia; B-LBL, B-cell lymphoblastic leukemia; MUD, matched unrelated donor; MA, myelo-ablative; NMA, non-myelo-ablative; PTLD, post-transplant lymphoproliferative disease. NCA, no cytogenetic abnormalities.

The study was approved by the central committee on research involving human subjects (CCMO), and the LUMC Institutional Review Board. From all patients and donors written informed consent was obtained. The primary objective of the study was to investigate the feasibility and safety of administration of donor derived HA-1H TCR-transduced virus-specific T cells after T-cell depleted alloSCT. Feasibility was defined as more than 50% of patients receiving at least one infusion of HA-1H TCR-transduced virus specific T cells posttransplant after inclusion in this study. Secondary objectives were to evaluate the persistence of HA-1H TCR-transduced virus-specific T cells after infusion, and to evaluate whether administration of HA-1H TCR-transduced virus-specific T cells makes patients eligible for standard donor lymphocyte infusion (DLI) at 6 months after alloSCT. Patients 18–75 years of age with high risk leukemia in complete or stable partial remission prior to transplant were eligible if they were HLA-A*02:01 and HA-1H positive, and transplanted with an HLA-matched HA-1H negative donor. The donor needed to be CMV and/or EBV seropositive allowing isolation of sufficient EBV or CMV-specific T cells. AlloSCT was performed as published previously (6, 17). HA-1H TCR-transduced virus-specific T cells were scheduled 8 and 14 weeks after alloSCT, since at that time points the alemtuzumab used in the conditioning regimen will not be circulating in the patient anymore. Contraindications for actual infusion of HA-1H TCR-transduced virus-specific T cells were acute GVHD overall grade II or higher or treatment with corticosteroids at a dose of 0.5 mg/kg prednisone or higher.

Flow Cytometry

Absolute numbers of circulating CD4 T, CD8 T, B, and NK cells were determined by the clinical Laboratory for Specialized Hematology (LUMC) as part of routine immune monitoring after transplantation on anticoagulated fresh venous blood using

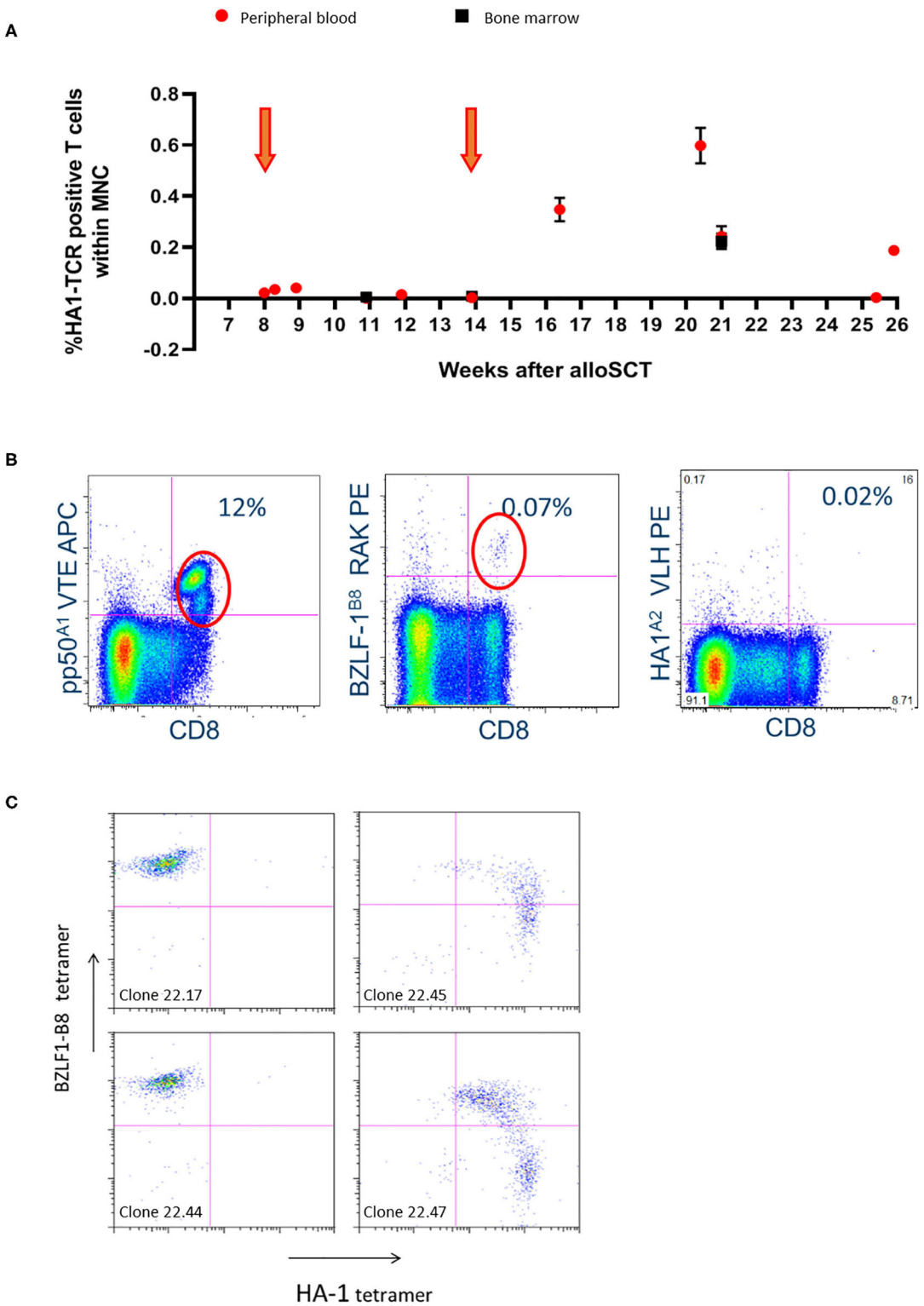

FIGURE 1 | Significant *in-vivo* persistence of HA-1H TCR-transduced T cells could be observed during follow-up with evidence of expansion after the second infusion in patient 001. **(A)** Vector-specific qPCR analysis was performed on peripheral blood and bone marrow samples at indicated time-points. Six weeks after the second infusion the highest peak of HA-1H TCR-transduced CMV or EBV-specific T cells peripheral blood and bone marrow samples was detected. Orange arrows illustrate infusion of HA-1h TCR modified T cells. **(B)** Facs analysis was performed on peripheral blood sample 6 weeks after infusion of the second cell line. Low numbers of EBV-specific T cells were observed (0.07%), and high frequencies of CMV-specific T cells were found including the infused CMV-pp50-A1-VTE specificity (12%). **(C)** T cells were isolated from PBMCs 6 weeks after second infusion using EBV-BZLF1-B8-RAK tetramers, single cell sorted and expanded, and tested after 14 days with the different pMHC-tetramers indicated. 50% of the EBV-BZLF1-B8-RAK-specific T-cell clones expressed the HA-1H TCR as measured by pMHC-tetramer analysis.

Trucount tubes (BD, Becton Dickinson, Breda, Netherlands) following the manufacturer's instructions. Samples were stained with allophycocyanin (APC)-conjugated CD3, PacificBlue-conjugated CD4, fluorescein isothiocyanate (FITC)-conjugated CD8, APC-H7-conjugated CD14, R-phycoerythrin (PE)-conjugated CD16, PE-Cy7-conjugated CD19, V500-conjugated CD45, and PE-conjugated CD56 (all from BD) antibodies and analyzed using a FACSCanto (BD).

Thawed PBMCs and bone marrow MNC cells from immunomonitoring samples and T-cell lines and clones were analyzed for binding to HA1H and virus-specific pMHC-tetramers by staining with PE- or APC-labeled pMHC-tetramers, and an Alexa700-conjugated antibody against CD8 (Invitrogen/Calteg, Buckingham, United Kingdom) combined with FITC-labeled antibodies against CD4, CD14, and CD19 (BD Pharmingen, San Jose, California). PBMCs and T-cell lines or clones were first incubated with 2 μg/ml pMHC-tetramers for 15 min at 37 °C before antibodies were added and incubated for an additional 15 min at 4°C. These analyses were performed on an LSRII (BD Biosciences, Franklin Lakes, New Jersey) and analyzed using Diva Software (BD Biosciences) or FlowJo Software (TreeStar, Ashland, Oregon).

Vector-Specific Real-Time Quantitative PCR of HA-1H TCR

Genomic DNA was isolated either using the AllPrep DNA/RNA/Protein mini kit (Qiagen) or QIAamp DNA Micro Kit (Qiagen) following manufacturer's instructions. Samples were run on a 7900HT RT-PCR System of Applied Biosystems. The following vector-specific HA1 TCR primers were used; forward primer resides in the optimized constant domain of the beta chain 5′ CTGTACGCCGTGCTGGTG 3′, reverse primer resides in the T2A region 5′ GGGATTCTCCTCCACGTCACC 3′ and the antisense probe also resides in the T2A region 5′ TGTTAGAAGACTTCCTCTGCCCTC 3′. The Probe used VIC as dye and TAMRA as quencher. Each sample was run in duplicate with 200 ng genomic DNA per well (qPCR core kit Eurogentec) at 65°C for 50 cycles.

T-Cell Reactivity Assays

T cells were cultured in T-cell medium (TCM) consisting of IMDM supplemented with 5% heat-inactivated FBS (Gibco, Thermo Fisher Scientific), 5% human serum, 3 mM L-glutamine (Lonza), 100 U/ml penicillin/streptomycin (Lonza), and 100 IU/ml IL-2 (Novartis). T-cell recognition was measured by IFN-γ ELISA (Sanquin) according to manufacturer's instructions. T cells (5,000 cells) were co-cultured with 30,000 cells (LCLs) or 60,000 cells (primary cells) in 60 μl TCM per 384 well flat bottom plates (Greiner Bio-One). Supernatants were harvested after overnight incubation to measure IFN-γ release. In peptide-pulsed conditions, stimulator cells were preincubated with 1 μM of the relevant peptide for 30 min at room temperature before addition to T cells.

Generation of T-Cell Clones

Virus-specific T cells were isolated from frozen PBMCs via FACS sorting using PE-labeled pMHC-tetramers. T cells were initially incubated with PE-labeled pMHC-tetramers for 1 h at 4°C and subsequently incubated with anti-CD8-FITC for 20 min at 4°C. pMHC-tetramer$^+$ CD8$^+$ T-cells were single-cell sorted into round-bottom 96-well plates containing 5×10^4 irradiated (35 Gy) allogeneic PBMNC as feeders in 100 μl T-cell medium supplemented with 0.8 μg/ml phytohemagglutinin (PHA; Oxoid Limited, Basingstoke, UK).

RESULTS

Feasibility and Characteristics of HA-1H TCR-Transduced CMV or EBV-Specific T-Cell Products

Nine donors were approached and gave consent to undergo leukapheresis to obtain PBMC for the generation of HA-1H TCR-transduced CMV or EBV-specific T-cell products. 2 donors were seropositive for both EBV and CMV, whereas 7 donors were only EBV seropositive. In 3 of these 7 donors frequencies of EBV-specific T cells with the appropriate specificity as measured by pMHC-tetramers were not above background, and therefore no EBV-specific T cells could be harvested, and no cell lines could be generated. From 6 donors, attempts were made to generate HA-1H TCR-transduced CMV or EBV-specific T cells (**Table 1**). From 5 donors a total of 10 HA-1H TCR-transduced CMV or EBV-specific T-cell products fulfilling the release criteria were generated (**Table 1**). Total cell numbers ranged from 3 to 283 $\times 10^6$ comprised of 96–99% T cells containing 74–100% virus-specific T cells and 11–41% HA-1H TCR-transduced T cells as measured by specific pMHC-tetramer staining. From donor UPG (patient 9) only very low numbers of EBV-specific T cells could be isolated, and at the end of the culture only 0.16×10^6 T cells were retrieved with only 5% of HA-1H TCR T cells, and therefore this product did not fulfill the release criteria. More detailed data about the composition of generated T-cell products is depicted in **Supplemental Figure 1**. These results illustrate that if virus-specific T cells could be detected in peripheral blood of the donors, in 5/6 cases HA-1H TCR- transduced CMV or EBV-specific T-cell products could be reproducibly generated.

Safety and Clinical Effect of Infusion of HA-1H TCR-Transduced CMV or EBV-Specific T Cells

Nine patients were included in the study. As illustrated in **Table 1** for 4 patients (patients 4, 6, 8, and 9) no HA-1H TCR-transduced CMV or EBV-specific T-cell product could be generated. Characteristics of patients who received a product are depicted in **Table 2**.

Four patients received the 2 scheduled infusions of the HA-1H TCR-transduced CMV or EBV-specific T cells, whereas patient 7 only received one dose. No immediate transfusion-related side effects were observed. Patient 1 showed persistent lymphopenia from the time of transplant until the end of follow-up. Significant *in-vivo* persistence of HA-1H TCR-transduced T cells could be observed by vector-specific PCR analysis during follow-up with evidence of expansion after the second infusion (**Figure 1A**). The patient developed antibody-mediated

TABLE 3 | Results of PCR measurements of HA-1H TCR transduced virus-specific T cells to evaluate persistence.

Weeks after 1st infusion	Weeks after 2nd infusion	HA-1H TCR positive T cells within total peripheral blood mononuclear cells (%)				
		Patient 001	Patient 002	Patient 003	Patient 005	Patient 007
0	−6	Undetectable	Undetectable	Undetectable	Undetectable	Undetectable
1	−5	0.041	Undetectable	Undetectable	$1 - 10^{-7}$	Undetectable
3	−3	0.001	Undetectable	Undetectable	$3 - 10^{-7}$	1.70
6	0	0.003	Undetectable	Undetectable	Undetectable	0.40
8	2	0.348	Undetectable	Undetectable	$3 - 10^{-7}$	0.52
12	6	0.598	Undetectable	Undetectable	Undetectable	
16	10	0.188	Undetectable	Undetectable	Undetectable	

neutropenia and thrombocytopenia 7 weeks after the infusion of the second cell line. Despite treatment with immunoglobulins, unmanipulated DLI, antibiotics and antifungal medication, the patient died from multiple viral, bacterial and fungal infections 19 weeks after the first infusion. In patients 2 and 3 no side effects occurred, no GVHD developed, and both patients received scheduled DLI 6 months after transplantation. At the time of last follow-up both patients are alive and well.

In patient 5, despite full donor chimerism as measured in peripheral blood, smoldering relapse was documented in the bone marrow aspirate at the time of the first infusion. The second infusion was performed as scheduled, but the patient developed progressive disease. Despite re-remission induction chemotherapy, no remission could be obtained, and the patient died from relapsed AML. No side effects related to the infusion were documented. Up to 3 weeks after the first and two weeks after the second infusion, low frequencies of HA-1H TCR-transduced CMV or EBV-specific T cells could be detected as measured by vector-specific PCR analysis, but no significant expansion of the cells could be documented (**Table 3**). This was not due to the inability of the relapsed AML to be targeted by HA-1H TCR-transduced CMV or EBV-specific T cells, since *in-vitro* analysis illustrated appropriate recognition of the leukemic cells (**Figure 2**) by these T cells.

In patient 7, from 3 weeks after infusion, expansion and persistence of HA-1H TCR-transduced T cells could be documented by vector-specific PCR in peripheral blood and bone marrow samples (**Figure 3A**), which coincided with an EBV reactivation. Five weeks after the infusion, limited signs of skin GVHD were observed, completely resolving after application of topical steroids. One week later the patient was admitted to the hospital with high fever caused by EBV-associated posttransplant lymphoproliferative disease (PTLD) with massive B-cell expansion. Despite the presence of EBV-specific T cells in peripheral blood these virus-specific T cells did not further expand, and despite treatment with rituximab and steroids, the condition rapidly deteriorated and the patient died from severe systemic disease.

In summary, for 5 out of 9 included patients HA-1H TCR-transduced CMV or EBV-specific T-cell products could be successfully generated and infused. In 3 patients persistence or expansion of HA-1H TCR-transduced CMV or EBV-specific T cells was detected. No transfusion-related toxicity was observed, and no significant GVHD developed after infusion. Three of the 5 patients died from complications probably not related to the infusion.

Persistence and Functionality of Infused HA-1H TCR-Transduced CMV or EBV-Specific T Cells

Significant *in-vivo* persistence and/or expansion of HA-1H TCR-transduced CMV or EBV-specific T cells was documented in patients 1 and 7 (**Figures 1A**, **3A**). From patient 1, 6 weeks after the second infusion at the highest peak of HA-1H TCR-transduced CMV or EBV-specific T cells (**Figure 1A**) peripheral blood and bone marrow samples were isolated. Low numbers of EBV-specific T cells were observed, but probably due to CMV reactivation high frequencies of CMV-specific T cells were found including the infused CMV-pp50-A1-VTE specificity (**Figure 1B**). However, no significant frequencies of HA-1H TCR-transduced T cells were detected as measured by pMHC-tetramer analysis (**Figure 1B**). To analyze whether HA-1H TCR-transduced T cells were present within these virus-specific T-cell populations, the CMV-pp50-A1-VTE and EBV-BZLF-1-B8-RAK positive T cells were FACS sorted using pMHC-tetramers. Vector-specific PCR analysis on the CMV-pp50-A1-VTE tetramer positive sorted cells demonstrated that only a very low frequency of HA-1H TCR-transduced T cells (0.01%) could be identified within the CMV-specific population. Apparently, after triggering by CMV antigen, non-HA-1H TCR-transduced CMV-specific T cells preferentially had expanded. In contrast, 50% of the EBV-BZLF1-B8-RAK-specific T-cell clones expressed the HA-1H TCR as measured by pMHC-tetramer analysis (**Figure 1C**) and vector-specific PCR analysis (data not shown).

In patient 7, significant persistence and expansion of HA-1H TCR-transduced EBV-specific T cells was observed (**Figure 3A**), probably provoked by exposure to EBV antigen as documented by the presence of circulating EBV antigen by PCR analysis. Despite the presence of EBV-specific T cells rapidly progressive donor-derived monoclonal EBV-associated PTLD developed. To determine why this PTLD could escape control by the circulating EBV-specific T cells, we analyzed *in-vitro* whether the PTLD could be recognized by EBV-EBNA3A-B7-RPP

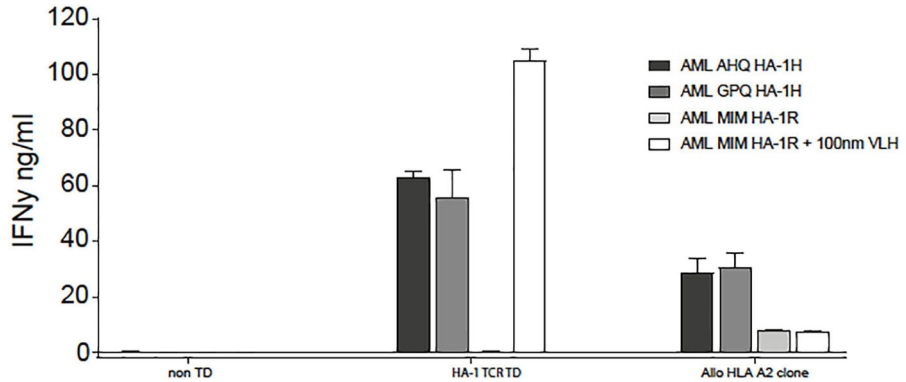

FIGURE 2 | HA-1H TCR-transduced T cells recognized HLA-A*02:01 positive, HA-1H positive primary AML cells, both patient AML cells (AHQ) at the time of relapse, as well as third party HLA-A*02:01 positive, HA-1H positive AML (GPQ) cells. HLA-A*02:01 positive, HA-1H negative AML (MIM) cells were only recognized if HA-1H peptide (VLH) was exogenously loaded on the AML cells.

or EBV-BZLF1-B8-RAK T-cell populations. As illustrated in **Figure 3B**, the PTLD was not recognized by these EBV-specific T-cell populations unless they were exogenously loaded with EBV-specific peptides. These results illustrate that the PTLD could escape control by the EBV-specific T cells due to absence of relevant endogenously processed viral antigens.

DISCUSSION

In this study we investigated whether HA-1H TCR-transduced CMV or EBV-specific T-cell products could be reproducibly generated from PBMCs of the stem cell donors seropositive for CMV or EBV. Our data illustrate that if sufficient virus-specific T cells could be isolated from the donor, HA-1H TCR-transduced CMV or EBV-specific T-cell products meeting the predefined release criteria could be generated. The drawback of the present study was that with the limited numbers of EBV or CMV- specific *Streptamer* products available, in several cases insufficient numbers of donor-derived virus-specific T cells could be harvested, especially in donors that were CMV seronegative implying that only EBV-specific T cells were available. This resulted in the successful production of the HA-1H TCR-transduced CMV or EBV-specific T-cell products ranging from 3 to 280 x 10^6 antigen-specific T cells for only 5 of 9 patients included in the study. The 5 HLA-A*02:01 and HA-1H positive patients with AML who were treated with the HA-1H TCR-transduced CMV or EBV-specific T-cells 8 and 14 weeks after T-cell depleted alloSCT did not experience infusion related toxicity, and no significant development or worsening of GVHD was observed. The complications observed in the patients after the infusions were considered not likely to be caused by the investigational product since at the time of these complications no significant expansion of the infused HA-1H TCR-transduced CMV or EBV-specific T cells was observed. From these data we concluded that although the infusions appear to be safe, the overall feasibility and efficacy of the procedure was too low to warrant further developments of this specific investigational product.

Several reasons may underlie the lack of expansion directly after infusion of HA-1H TCR-transduced CMV or EBV-specific T cells. As demonstrated in clinical trials using CAR T cells for the treatment of relapsed or refractory hematological malignancies, pre-conditioning with *in-vivo* lymphodepleting chemotherapy appears to be essential for significant expansion of infused cells probably related to the *in-vivo* induction of lymphocyte activating interleukins and depletion of regulatory T cells (37–39). Since in our study we scheduled to treat patients in remission in a prophylactic setting, no lymphodepleting conditioning could be applied. Secondly, we hypothesized that co-expression of the virus specific TCR and the HA-1H specific TCR-transduced T cells would lead to expansion when exposed to either viral antigens or the HA-1H antigen (34, 35). The *in-vivo* data suggest that in the absence of significant exposure to recipient-derived HA-1H antigen expressing hematopoietic cells, expansion of T cells co-expressing both TCRs did not or hardly occur. We hypothesize that by codon optimization and cysteine modification of the HA-1H TCR, and selection of virus specific T cells with a weak competitor phenotype we created an HA-1H TCR that successfully competed for membrane expression with the endogenous virus-specific TCR, resulting in lack of expansion in the presence of only viral antigens (34, 35, 40). This has likely resulted in the expansion of mostly the non-transduced virus-specific T cells from the infused product during viral reactivation after transplantation. As a consequence, in only 2 patients significant persistence and expansion of HA-1H TCR-transduced CMV or EBV-specific T cells could be illustrated, correlating with a viral reactivation. Only in the patient who showed smoldering relapse at the time of infusion, low frequencies of HA-1H TCR-expressing cells were present, but also under these circumstances, the malignant cells outgrew the HA-1H TCR-transduced CMV or EBV-specific T cells. There appeared to be no HA-1H antigen escape variant in this patient, since also the relapsed AML cells were shown to be recognized by HA-1H TCR-transduced CMV or EBV-specific T cells *ex-vivo*.

In one patient, lethal PTLD developed in the presence of EBV-specific T cells with specificities that were also present in the infused investigational product. Further analysis illustrated these

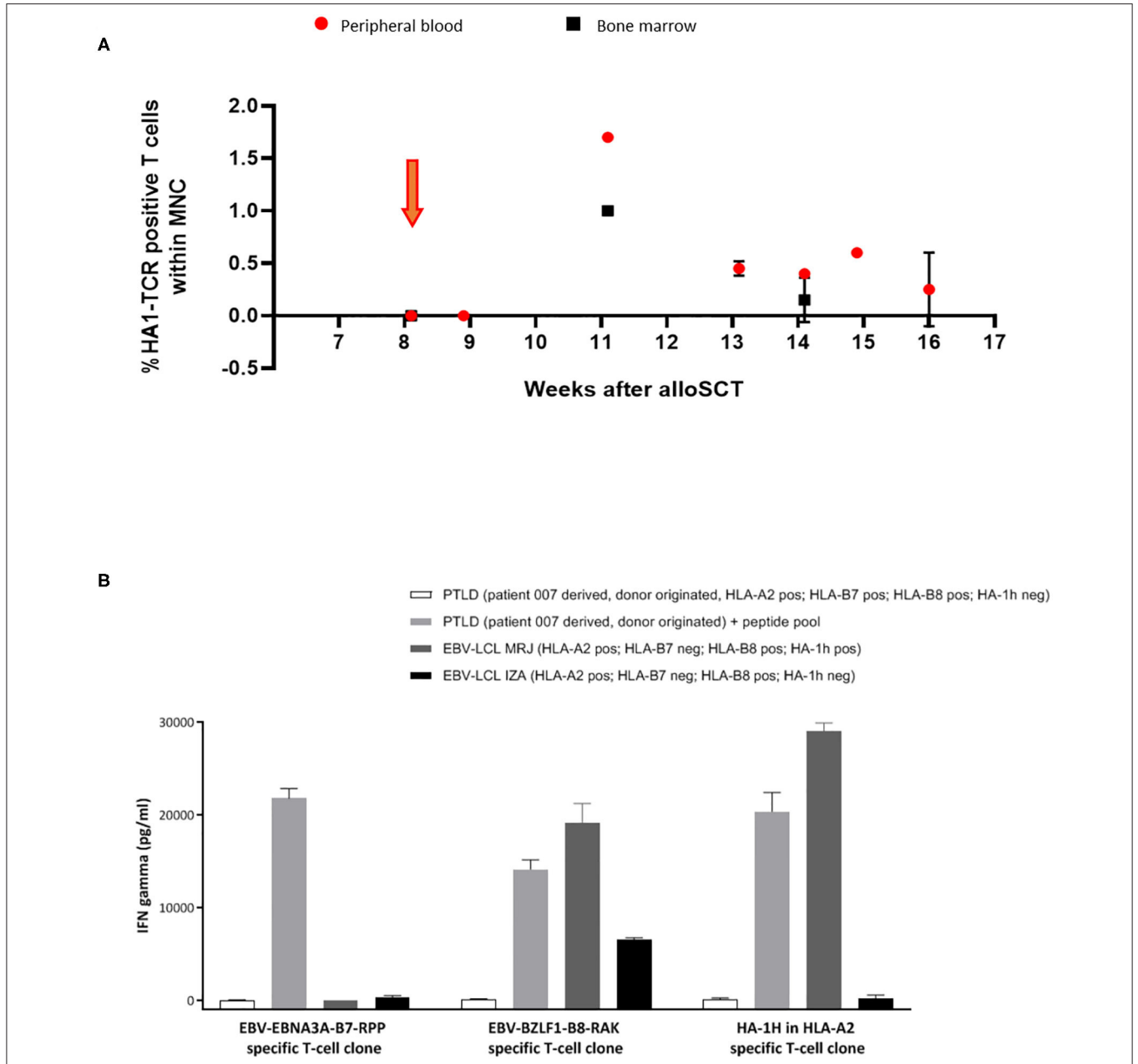

FIGURE 3 | Significant expansion and persistence of HA-1H TCR-transduced T cells could be documented in peripheral blood and bone marrow samples of patient 007. **(A)** Vector -specific qPCR analysis was performed on peripheral blood and bone marrow samples at indicated time-points. Orange arrow illustrates infusion of HA-1H TCR-modified T cells. **(B)** EBNA-3A-B7 and BZLF1-B8 and HA1H specific T cells were stimulated with patient 007 PTLD (SLC PTLD) that was not loaded or loaded with pool of the 2 EBV peptides, LCL-MRJ (HLA-A2+, B7-, B8+, HA-1H+, BZLF1+), and LCL-IZA (HLA-A2+, B7-, B8+, HA-1H-, BZLF1+). PTLD of patient 007 was not recognized by EBV-specific T-cell populations unless they were exogenously loaded with EBV-specific peptides. This PTLD was also not recognized by HA-1H-specific T cells, because the PTLD was donor-derived and therefore HA-1H negative. PTLD sample consisted of monoclonal B cells after CD19 enrichment of PBMNC.

EBV-positive clonal B cells of donor origin were transformed not to express the antigen specificities targeted by the circulating EBV-specific T cells. No significant numbers of B cells were present in the investigational product, and therefore we concluded that this complication was not due to the experimental treatment and that lack of control by the T cells present in the product was caused by an antigen negative variant which has been found more frequently in patients with monoclonal PTLD.

In conclusion, we have demonstrated that HA-1H TCR-transduced CMV or EBV-specific T-cell products can be reproducibly made if sufficient virus-specific T cells can be isolated from virus seropositive donors. Infusion of these

products into patients with high-risk AML appears to be safe, but overall feasibility and efficacy of this approach appears to be too low to allow further development of this investigational product. A new strategy will be explored using products consisting of donor-originated CD8 T cells isolated from the patient after transplantation and transduced with the HA-1H TCR gene to be infused following immune conditioning in patients with persistent or relapsed hematological malignancies after HA-1H-mismatched transplantation. A new clinical trial has recently been approved (EudraCT 2019-002346-20) that implemented several improvement to the limitations of the strategy followed in this manuscript. The improvements aim to overcome the identified weaknesses of lack of lymphodepleting condition and lack of HA-1H antigen expression in the recipient. Transduction of not only virus-specific T cells but all donor derived CD8 T cells circulating in allogeneic transplanted patients and infusion of a targeted dose of HA-1H TCR transduced T cells will result in higher numbers of infused T cells. Again, HA-1H TCR transduction is preferred over using peptide stimulated HA-1H specific T cells since that method is much more time consuming and laborious without expected better efficacy. This will allow further evaluation of the potential efficacy of MiHA-TCR-transduced T-cell products in the treatment of hematological malignancies in the context of alloSCT.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Centrale Commissie Mensgebonden Onderzoek (CCMO). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

PB, IJ, ML, HV, JF, and MH designed the research, analyzed results, and wrote the paper. ML, RB, HE, RH, CH, SV, LH, PL, J-JZ, PM, and IJ performed experiments and were responsible for manufacturing the T-cell products. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01804/full#supplementary-material

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