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Rapid Identification of Clinical Relevant Minor Histocompatibility Antigens via Genome-Wide Zygosity-Genotype Correlation Analysis

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Abstract

Purpose: Identification of minor histocompatibility antigens (mHag) with classic methods often requires sophisticated technologies, determination, and patience. We here describe and validate a nonlaborious and convenient genetic approach, based on genome-wide correlations of mHag zygosities with HapMap single-nucleotide polymorphism genotypes, to identify clinical relevant mHags within a reasonable time frame.

Experimental Design: Using this approach, we sought for the mHag recognized by a HLA-DRB1*1501–restricted T-cell clone, isolated from a multiple myeloma patient during a strong graft-versus-tumor effect associated with acute graft-versus-host disease grade 3.

Results: In a period of 3 months, we determined the mHag phenotype of 54 HapMap individuals, deduced the zygosity of 20 individuals, defined the mHag locus by zygosity-genotype correlation analyses, tested the putative mHag peptides from this locus, and finally showed that the mHag is encoded by the arginine (R) allele of a nonsynonymous single-nucleotide polymorphism in the *SLC19A1* gene.

Conclusions: We conclude that this powerful and convenient strategy offers a broadly accessible platform toward rapid identification of mHags associated with graft-versus-tumor effect and graft-versus-host disease. (Clin Cancer Res 2009;15(23):7137–43)

HLA-matched allogeneic stem cell transplantation (allo-SCT) is an effective treatment for hematologic malignancies. After allo-SCT, donor T cells directed at the minor histocompatibility antigens (mHag) of the recipient mediate graft-versus-tumor (GvT) effects, but they can also cause graft-versus-host disease (GvHD). mHags are polymorphic peptides derived from intracellular proteins and presented by HLA molecules (1–4). Pioneering studies indicated that mHags expressed exclusively in hematopoietic cells can serve as excellent tools to separate GvT effects from GvHD (5). Currently, one of the important bottlenecks toward broad application of mHag-based immunotherapy strategies is the speed of identifying relevant hematopoietic mHags. Classic identification methods such as peptide elution and cDNA library screening are complex, time-consuming, and offer a moderate chance of success (3, 4, 6). Recently introduced pairwise linkage analyses are also time-consuming, require advanced genetic know-how, and are not always successful in identifying the precise mHag locus (7–10). We recently identified the $CD19^{L}$-encoded mHag by combining pairwise linkage analysis with a novel fine-mapping strategy, called zygosity-genotype correlation analysis (11). In retrospective computational analyses, the genome-wide approach of zygosity-genotype correlation analysis seemed powerful enough to be applied as stand-alone identification strategy for mHags with 10% to 85% population frequency. Because this method was hypothesized to be more rapid and accessible than other (genetic) approaches, we now explored its actual speed and ease, using a $CD4^{+}$ T-cell clone recognizing a novel HLA-DRB1*1501 restricted mHag. Within only 3 months, we succeeded in identifying the HLA class II mHag as a polymorphic peptide encoded by the $SLC19A1^{R}$ gene.

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Materials and Methods

Cells. The HLA-DRB1*1501 restricted $CD4^{+}$ T-cell clone 1GF5 was previously isolated from a multiple myeloma patient (12). It was expanded using a feeder cell cytokine mixture as previously described (13). EBV-transformed lymphoblastoid cell lines (EBV-LCL) and the Phoenix packaging line were cultured in RPMI 1640 and DMEM (Invitrogen), respectively, both supplemented with 10% fetal bovine serum (Integro) and antibiotics.

T-cell–mediated cytotoxicity assay. Serial dilutions of effector T-cell clone 1GF5 were incubated with luciferase-transduced multiple myeloma

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Translational Relevance

In this study, we have identified a new autosomal HLA class II restricted minor histocompatibility antigen using a new genetic approach in a time frame of only months. The identified mHag, SLC19A1^R, will be frequently mismatched in an allogeneic stem cell transplantation and is likely to be involved in graft-versus-tumor effect and graft-versus-host disease. Thus, our genetic approach can facilitate rapid and large-scale molecular characterization of novel clinically relevant mHags. Toward meaningful exploitation of our approach, we determined a directed strategy and calculated the number of mHags for wide-scale application of mHag-specific immunotherapy. We included both HLA class I and HLA class II restricted mHags because not only CD8$^+$ but also CD4$^+$ T cells contribute to antitumor reactions after allogeneic stem cell transplantation. Thus, rapid identification of mHags with our technology and following a directed strategy are relevant approaches toward wide-scale clinical application of mHag-specific T-cell immunotherapy.

cell lines in the presence of 125 μg/mL beetle luciferin (Promega) in white opaque flat-bottomed 96-well plates (Costar). After 6 h, the light signal emitted from surviving multiple myeloma cells was determined using a luminometer (Molecular Devices), and the percentage lysis was calculated compared with medium control (set to 0%) as described (14).

Retroviral vectors and virus production. The pMX-HLA-DRB1*1501-IRES-GFP and the pMX-SLC19A1^{27R}-IRES-GFP vectors were generated by cloning commercially synthesized genes, *HLA-DRB1*1501* and *SCL19A1^{27R}* (GenScript), into the pMX-vector as described (13). Generation of retroviral supernatants and retroviral transductions were described elsewhere (13).

SLC19A1-derived peptides. Commercially synthesized and purified 15-mer peptides (Pepscan) were dissolved in DMSO to 100 mmol/L and diluted in PBS to 6 mmol/L for use in functional assays.

mHag phenotyping of HapMap EBV-LCLs. The phenotyping procedure has been described previously (11). In short, HLA-DRB1*1501–positive (naturally positive or positive after retroviral transduction) EBV-LCLs from HapMap individuals were used as antigen-presenting cells to stimulate T-cell clone 1GF5. IFN-γ release in supernatants was determined using ELISA (Invitrogen). EBV-LCLs were judged mHag$^+$ if the mean absorbance value at 450 nm of triplicate cultures was >0.250, about three times the background absorbance value, as described (11).

Genome-wide zygosity-genotype correlation analysis. mHag zygosities (+/+, +/-, or -/-) of HapMap individuals were deduced from the Mendelian segregation pattern of the mHag phenotypes in the father-mother-child trios as previously described (11). Zygosity-genotype correlation analysis was done using a modified version of the open source software ssSNPer (15) with embedded HapMap single-nucleotide polymorphism (SNP) genotypes for the CEU population[4] (16, 17). The complete Linux-based analysis package is available online for download.[5] Information about the *SLC19A1* gene and relevant SNPs was derived from Ensembl.[6]

[4] Downloaded from http://www.hapmap.org/.
[5] http://www.umcutrecht.nl/subsite/dcch/Research/Hemato-Oncology/
[6] http://www.ensembl.org/

Results

Selection of the mHag-specific T-cell clone. To validate the genome-wide zygosity-genotype correlation approach (Fig. 1) and to evaluate its speed, we used the HLA-DRB1*1501 restricted mHag-specific CD4$^+$ T-cell clone 1GF5. The HLA class II restriction of this clone was ideal for validation of our approach because identification of HLA class II restricted mHags is usually more difficult as compared with HLA class I restricted antigens. Furthermore, the mHag could have clinical relevance because the clone 1GF5 was isolated from a multiple myeloma patient during a strong GvT effect associated with acute GvHD grade 3 and showed high IFN-γ release as well as strong cytotoxic activity against an allogeneic multiple myeloma cell line in a mHag-specific fashion (Fig. 2A and B).

Mapping of the mHag recognized by 1GF5 via mHag zygosity–based genome-wide analysis. The genetic correlation analyses in our approach use mHag zygosities, which can be deduced using inheritance of mHag phenotypes. Therefore, to identify the mHag recognized by 1GF5, we first determined the mHag phenotypes of several HapMap trios (father-mother-child) by testing the reactivity of 1GF5 toward EBV-LCLs derived from these individuals. In total, we phenotyped 54 EBV-LCLs, of which 42 (78%) were mHag positive (Table 1). Using these data, we could deduce the mHag zygosity (+/+, +/-, or -/-) of 20 of 54 individuals (see Table 1 and Fig. 1 for an example). Correlating this zygosity information to the genotypes of almost four million SNPs in the whole genome using a freely available software[7] revealed 100% correlation (r^2 = 1) with four SNPs (rs3788200, rs1051266, rs4819130, and rs1131596; Fig. 3A), located within one linkage disequilibrium block on chromosome 21. This indicated that this linkage disequilibrium block could contain the SNP encoding for the mHag. Supporting this idea, the four SNPs also showed 100% correlation with the mHag phenotypes of the remaining 34 mHag$^+$ EBV-LCLs whose mHag zygosities could not be deduced.

Identification of the SLC19A1^R-encoded mHag. One of the four 100% correlating SNPs (rs1051266) was nonsynonymous, encoding for a histidine (H) to an arginine (R) substitution at position 27 of SLC19A1 (Fig. 3B), suggesting that SLC19A1^R could be encoding the mHag. Supporting this possibility, the original SCT patient, but not the donor, carried the SLC19A1^R-encoding allele (data not shown), and donor EBV-LCLs were recognized by 1GF5 on transduction with the *SLC19A1^R* gene but not with an empty vector (Fig. 3C). Finally, 1GF5 recognized several synthetic 15-mer peptides containing the SLC19A1^R-derived sequence **R**LVCYLC, illustrating that the HLA class II restricted mHag was indeed a polymorphic peptide derived from SLC19A1^R (Fig. 3D). Analysis of all other CD4$^+$ mHag-specific T-cell clones isolated from the patient revealed the recognition of SLC19A1^R peptides by five additional clones using at least two different T-cell receptors. All these clones also recognized the mHag$^+$ multiple myeloma cell line UM9 (data not shown).

Discussion

In this study, we describe and validate a nonlaborious and convenient genetic approach to identify clinical relevant mHags. Using this genome-wide zygosity-genotype correlation

[7] http://www.umcutrecht.nl/subsite/dcch/Research/Hemato-Oncology

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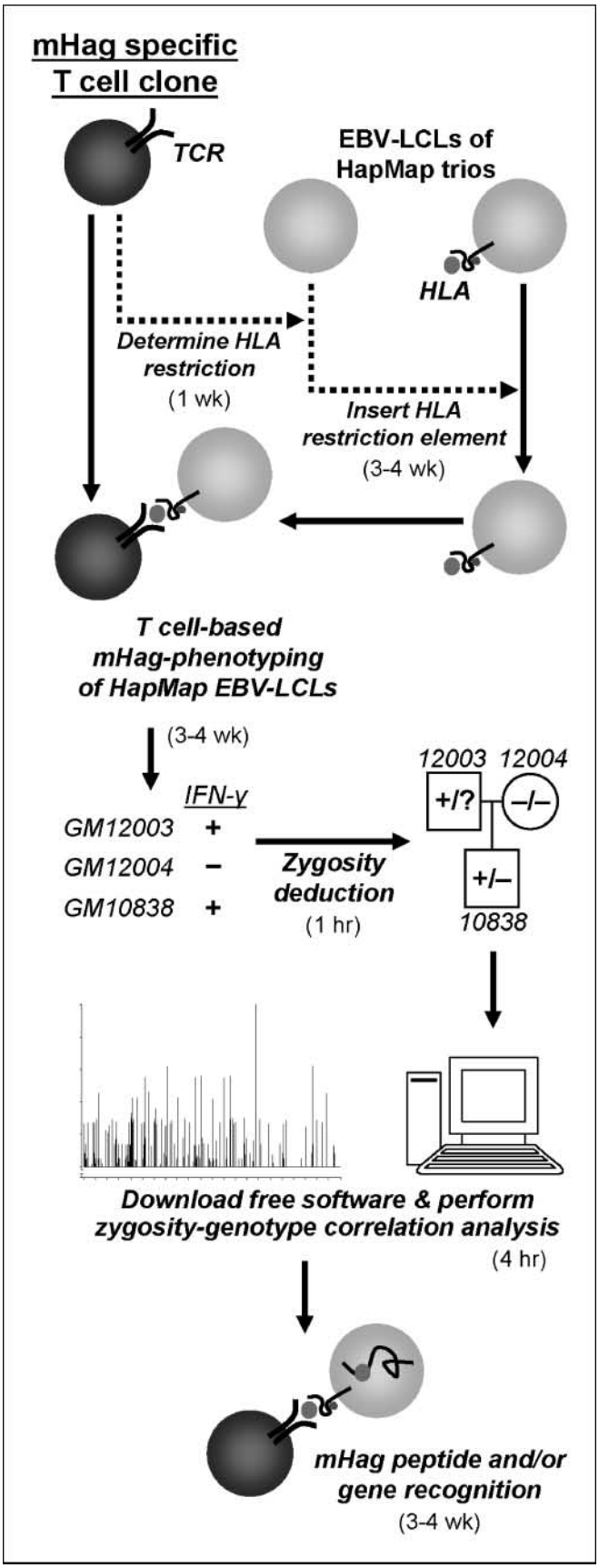

Fig. 1. Schematic overview of the genome-wide mHag identification strategy. The approximate time needed for each single step is indicated.

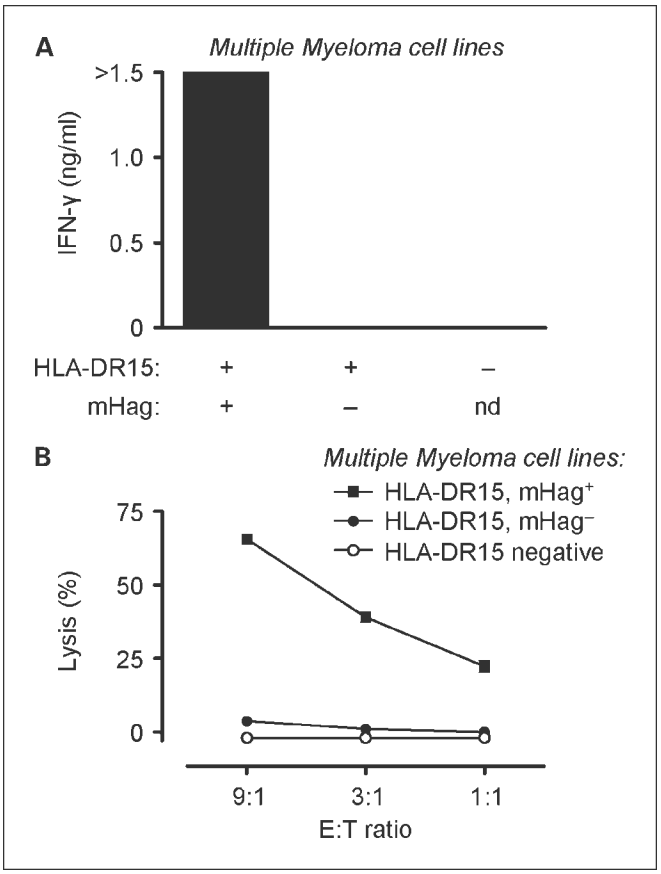

Fig. 2. 1GF5 recognizes and lyses multiple myeloma cell line UM9. IFN-γ response (*A*) and cytotoxic activity (*B*) of 1GF5 against three multiple myeloma cell lines [UM9 (HLA-DRB1*1501, mHag$^+$), U266 (HLA-DRB1*1501, mHag$^-$), and RPMI (HLA-DRB1*1501 negative)] after 18 h (*A*) and 6 h (*B*) of coincubation using different effector-to-target (*E:T*) ratios. Columns and points, average of triplicate cultures, which are representative of two independent experiments; bars, SEM.

approach, we identified the mHag recognized by a CD4$^+$ T-cell clone as a polymorphic peptide derived from the *SLC19A1*^{R} gene, which encodes a transmembrane protein functioning as a transporter for natural folate compounds (18). The SNP encoding for the SLC19A1^R mHag is polymorph in all HapMap populations, with a phenotype frequency between 39% and 88%,[8] implying that a mismatch for this new mHag will be frequently encountered in an allo-SCT setting. It is likely that this HLA class II restricted mHag can contribute to the GvT effect after allo-SCT because multiple SLC19A1^R mHag-specific T cells were isolated from our patient who manifested a strong GvT effect, and all of these T cells were capable of recognizing a mHag$^+$ multiple myeloma cell line. To date, two other autosomal HLA class II restricted mHags are known: the ubiquitously expressed PI4K2B^S mHag presented by HLA-DQ6 and the hematopoietic restricted CD19^L mHag presented by HLA-DQ2 (11, 19). Both mHags are suggested to be involved in GvT effects. On the other hand, because microarray data[9] indicate that SLC19A1 is expressed not only in hematopoietic cells but also in lung and liver, the mHag derived from SLC19A1 may also contribute to the development of GvHD after allo-SCT.

[8] http://www.hapmap.org/
[9] http://biogps.gnf.org/

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In a retrospective evaluation, we determined that the whole mHag identification procedure took only 3 months. It should be stressed that the whole procedure for the identification of the SLC19A1^{R} mHag was extremely rapid because one of the SNPs identified by the correlation analysis encoded for the searched mHag. We think that such cases will be encountered frequently because HapMap includes 25% to 35% of common SNP variation in the human genome (17). However, even if the

Table 1. Phenotyping and zygosity determination of HapMap individuals

HapMap individual	Trio relation	IFN-γ absorbance value	Phenotype	Zygosity
GM12144	Father	1.309	+	
GM12145	Mother	1.936	+	
GM06994	Father	0.053	-	-/-
GM07029	Child	1.425	+	+/-
GM07022	Father	0.946	+	
GM07019	Child	1.227	+	
GM06993	Father	0.138	-	-/-
GM06991	Child	0.051	-	-/-
GM07034	Father	1.113	+	
GM07055	Mother	1.761	+	
GM07048	Child	0.951	+	
GM12056	Father	1.897	+	
GM12057	Mother	0.903	+	
GM12043	Father	1.479	+	
GM12044	Mother	1.874	+	
GM10857	Child	1.538	+	
GM11881	Father	1.478	+	
GM11882	Mother	1.900	+	
GM10859	Child	0.635	+	
GM11839	Father	1.913	+	
GM11840	Mother	1.562	+	
GM10854	Child	1.331	+	
GM11831	Father	1.733	+	
GM11832	Mother	1.673	+	
GM10855	Child	0.403	+	
GM11829	Father	0.986	+	
GM11830	Mother	0.824	+	
GM10856	Child	1.139	+	
GM11992	Father	1.334	+	
GM11993	Mother	1.983	+	
GM10860	Child	1.294	+	
GM11994	Father	1.551	+	
GM11995	Mother	1.366	+	
GM10861	Child	1.608	+	
GM12264	Father	0.064	-	-/-
GM10863	Child	1.401	+	+/-
GM12154	Father	1.720	+	+/-
GM12236	Mother	0.060	-	-/-
GM10830	Child	0.056	-	-/-
GM12155	Father	1.090	+	+/-
GM12156	Mother	0.545	+	+/-
GM10831	Child	0.052	-	-/-
GM12248	Father	0.074	-	-/-
GM12249	Mother	1.186	+	+/-
GM10835	Child	0.048	-	-/-
GM12003	Father	1.534	+	
GM12004	Mother	0.068	-	-/-
GM10838	Child	1.288	+	+/-
GM12005	Father	1.456	+	
GM10839	Child	1.234	+	
GM12750	Father	1.369	+	
GM12761	Mother	0.060	-	-/-
GM12752	Child	1.459	+	+/-
GM12815	Mother	0.071	-	-/-

NOTE: Depicted are the 54 phenotyped HapMap individuals derived from father-mother-child trios. The IFN-γ response of clone 1GF5 (average absorbance value of triplicate culture) against their EBV-LCLs (naturally HLA-DRB1*1501 positive or retrovirally transduced) is shown with the subsequently designated phenotypes (absorbance value >0.250 was considered as mHag^{+}; ref. 11). The mHag zygosity was deduced from the inheritance pattern of the phenotypes within each trio (see Fig. 1 for an example). For some individuals, trios did not provide sufficient information to deduce the mHag zygosity (empty fields). The analysis depicted in Fig. 3A was executed only with those individuals for whom the mHag zygosity information was available.

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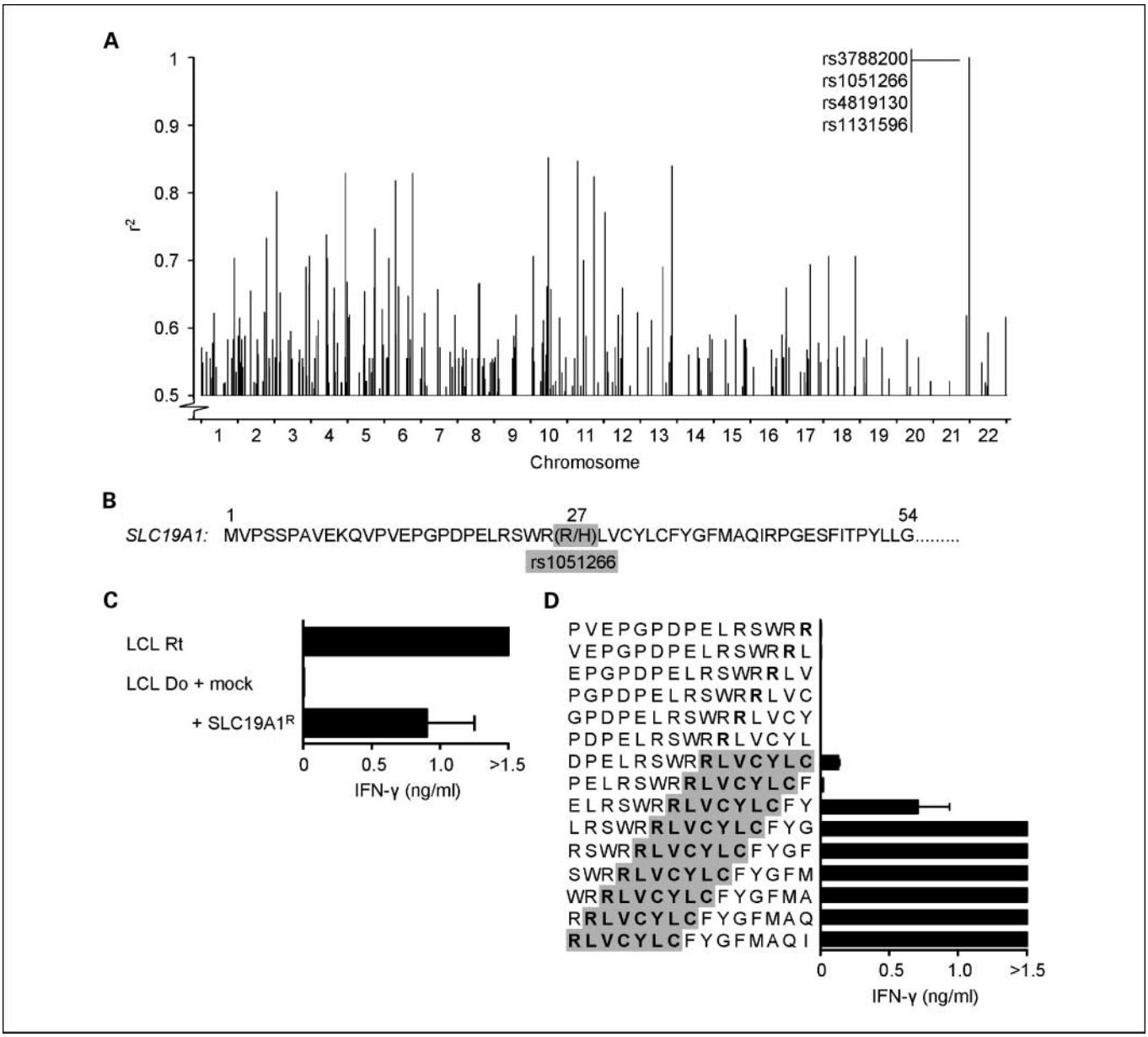

Fig. 3. Genome-wide mapping of the SLC19A1^R-encoded mHag recognized by 1GF5. *A,* zygosity-genotype correlation analysis was done with the 20 known mHag zygosities shown in Table 1. Each column represents a single SNP. The r^2 values represent the correlation between the mHag zygosities of the EBV-LCLs and their genotypes for ~4 × 10^6 HapMap SNPs. Only r^2 values above 0.5 are shown. The four SNPs (rs3788200, rs1051266, rs4819130, and rs1131596) with 100% correlation (r^2 = 1.0) are indicated. *B,* the segment of SLC19A1 in which the SNP rs1051266 encodes for a histidine (H) to an arginine (R) substitution at amino acid position 27. *C,* IFN-γ response (arbitrary units) of 1GF5 to mHag$^-$ donor (*Do*) EBV-LCLs (*LCL*) transduced either with an empty vector (*mock*) or with the full-length SLC19A1^R-encoding vector. Response to mHag$^+$ recipient (*Rt*) EBV-LCLs is depicted as positive control. *D,* IFN-γ response of 1GF5 toward donor EBV-LCLs loaded with SLC19A1^R-derived overlapping 15-mer peptides. *C* and *D,* horizontal columns, mean of triplicate wells; bars, SEM. Representative results from two different experiments.

searched SNP is not identified directly, additional exploration may not take too long because the regions identified with this method usually contain only a few candidate genes (11, 20). Thus, the strategy is indeed much faster than many other strategies described thus far and therefore likely to overcome the most important bottleneck toward efficient identification of clinical relevant mHags. Technically, in our strategy, it is important to realize that errors in mHag phenotyping will negatively influence the outcome of the analysis. Although our previous calculations indicate that 10% of phenotyping errors can be tolerated, the analyses will become more complicated, and therefore phenotyping should be done with great care. Still, the correlation analyses may fail in ~7% of the cases because HapMap estimates that their SNPs can currently capture ~93% of all SNPs with minor allele frequency of >0.05 within CEU populations (17). Furthermore, our approach may be less suitable for the identification of mHags that are only frequent in limited ethnical populations because our approach is based on the use of trios, and the International HapMap Project does not include trios for all ethnicities.

With the rapid identification of mHags now possible, the remaining question is "How many new mHags presented by

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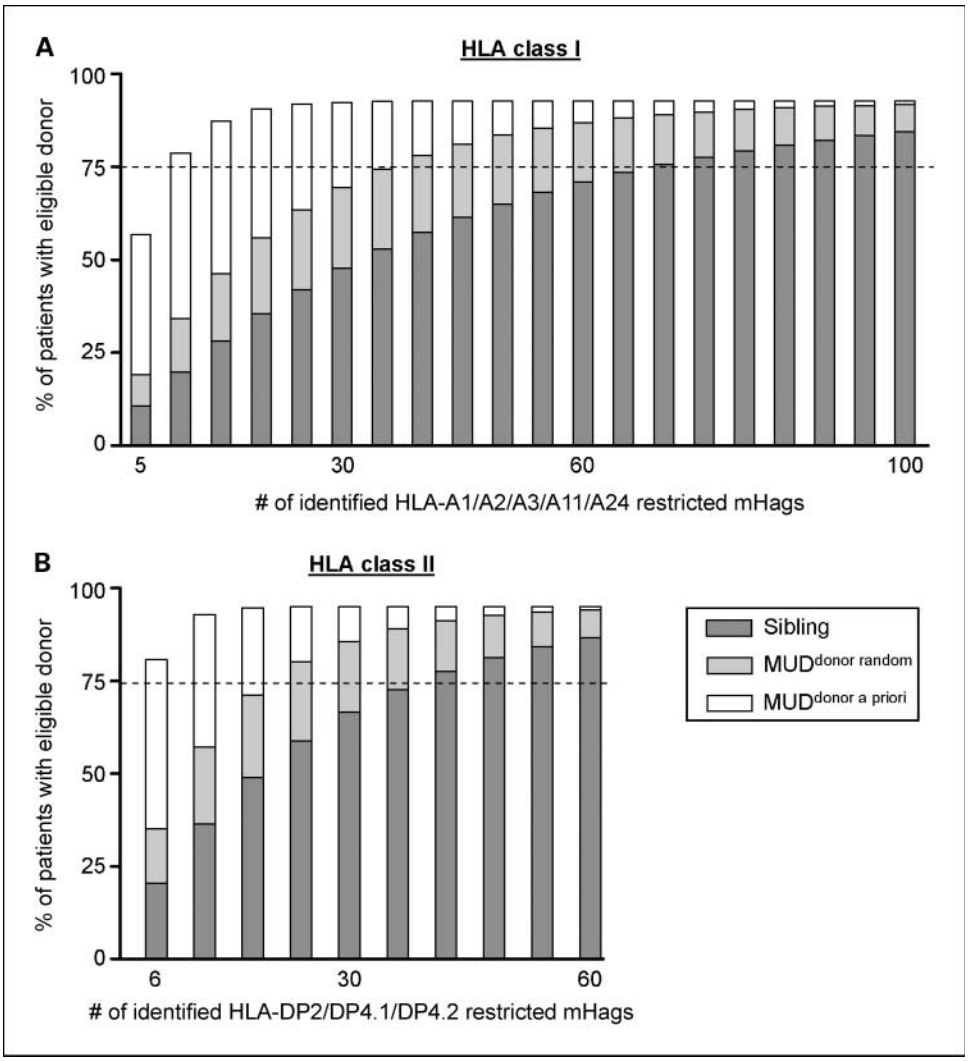

Fig. 4. The coverage of the European population eligible for mHag-based immunotherapy. The histogram represents the coverage of European patients eligible for mHag-based immunotherapy (patient mHag$^+$/donor mHag$^-$) per five or six mHags each presented by respectively one of the most frequent (*A*) HLA-A alleles (HLA-A1, HLA-A2, HLA-A3, HLA-A11, HLA-A24) or (*B*) HLA-DP alleles (HLA-DPB1*0201, HLA-DPB1*0401, HLA-DPB1*0402). Coverage percentages are shown for sibling transplantation (*dark gray*), random MUD transplantation (*dark gray plus light gray*), or transplantation from an a priori MUD (*dark gray plus light gray plus white*). Calculations were based on three assumptions: (*a*) The identified mHags will have a population frequency of 10% to 85% (limits of our strategy; ref. 11). (*b*) Frequency distribution of mHags is similar to that of SNPs described by HapMap (phase I + II; ref. 17). (*c*) mHags are randomly identified and not linked to each other. The mHag mismatch chances for sibling and MUD transplantations were calculated as described (11). HLA coverage for the European population was calculated using a published online tool (22). The dashed line is drawn at 75%.

which HLA molecules should be identified for wide-scale application of mHag-based immunotherapy?" Several mHags identified thus far have little significant clinical value because they are presented by infrequent HLA alleles (6, 21). It seems easy to tackle this issue by focusing on frequent HLA-A and HLA-B alleles. However, focusing on both HLA types does not increase population coverage significantly because frequent HLA-A and HLA-B alleles are inherited together as common haplotypes (22, 23). Therefore, focusing either on the most frequent HLA-A alleles or on the most frequent HLA-B alleles is a better strategy to make significant progress. HLA-A alleles seem a better choice for different populations. For instance, it is known that >92.5% of the European population expresses at least one of the HLA-A alleles (HLA-A1, HLA-A2, HLA-A3, HLA-A11, and HLA-A24; ref. 22). In contrast, more than 10 HLA-B alleles are necessary to achieve a similar coverage in the same population. Similarly, for HLA class II alleles, >95% of the population is covered by only three HLA-DP alleles (HLA-DPB1*0201, HLA-DPB1*0401, and HLA-DPB1*0402). We calculated that approximately 14 mHags per aforementioned HLA-A or HLA-DP allele are needed to establish at least one mHag mismatch in >75% of sibling transplantations (Fig. 4A and B). This number decreases to eight in case of a matched unrelated donor (MUD) setting and only to a couple if MUDs could be a priori selected for a mHag mismatch (Fig. 4A and B). Thus, broad application of mHag-based strategies will be possible with, in total, ~40 HLA-A or ~24 HLA-DP restricted mHags, and we believe that our identification strategy can significantly contribute to achieve this goal.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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