Supporting information for:

An unexplored angle: T cell antigen discoveries reveal a marginal contribution of proteasome splicing to the immunogenic MHC class I antigen pool

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Figure S1. Identification of hematopoietic minor histocompatibility antigens by GWAS. For identification of minor H antigens, T cell clones were evaluated for their reactivity against a panel of 191 EBV-LCLs (IFN-γ release, X-axis). SNPs in these EBV-LCLs were sequenced and genotyped by the 1000 Genomes Project. Two example HLA-B*07:02-restricted T cell clones for which the cognate minor H antigens were identified in the ensuing GWAS with the lowest (top panel) and highest (bottom panel) observed p-values are displayed. EBV-LCLs negative for HLA-B*07:02 were excluded from the GWAS (top within each panel). Results are shown for associating SNPs encoding the minor H antigens. EBV-LCLs that encode the minor H antigen encoding SNP allele are shown by red (GG in top and CC in bottom panel) and yellow (AG in top and AC in bottom panel) dots, whereas blue dots represent EBV-LCLs that harbored the negative allele (AA).
Table S1. List of all reported HLA class I restricted minor H antigens identified using forward antigen identification strategies.

| Spliced minor H antigens |   |   |   |   |   |
|--------------------------|---|---|---|---|---|
| peptide                  | antigen name | HLA class I restriction | method          | reference |
| [SLPRGT][STPK]           | SP110         | HLA-A*03:01              | cDNA library    | (1)       |

| Non-spliced minor H antigens |   |   |   |   |   |
|-----------------------------|---|---|---|---|---|
| peptide                    | antigen name | HLA class I restriction | method | reference |
| RTLSPEREITV                | LB-WNK1-1I   | HLA-A*02:01              |        | (2)       |
| SLAVAQDLT                  | LB-SSR1-1S   |                        |        |           |
| FMWDVAEDLKA                | LB-PRCP-1D   |                        |        |           |
| HPRQEIQALLA                | LB-ERAP1-1R  |                        | GWAS   |           |
| QPRRALLFVIL                | LB-BCAT2-1R  |                        |        |           |
| LPRACWREA                  | LB-ARHGDB1-1R|                        |        |           |
| GPDSSKTFCL                 | LB-PDCD11-1F |                        |        |           |
| FPALRFVEV                  | LB-GEMIN4-1V |                        |        |           |
| RPRARYIQV                  | LB-EBI3-1I   |                        |        |           |
| KPOQYHAEMCF                | LB-APOBEC3B-1K|                      |        |           |
| WLQGVPVPV                  | LB-NCAPD3-1Q |                        |        |           |
| HLEEOQPKV                  | LB-NDC80-1P  |                        |        |           |
| CADPSETWV                  | LB-CCL4-1T   |                        |        |           |
| RPCPSVGLSFL                | LB-C16ORF1-1R|                      |        |           |
| RPRSVTIQPLL                | LB-TMEM8A-1I |                        |        |           |
| RPRYWILLVKI                | LB-ZDHHC6-1Y |                      |        |           |
| CPRPGTWTC                  | LB-MOB3A-1C  |                        |        |           |
| FPALRFVEV                  | LB-GEMIN4-2V |                        |        |           |
| DLRCKYVSL                  | LB-GSTP1-1V  |                        |        |           |
| KPOQYHAEMCF                | LB-APOBEC3B-2K|                     |        |           |
| TQAQIFDYSEI                | LB-PNP-1S    |                        | HLA-B*13:01  |           |
| NEIEDWQYLDL                | LB-ZNFX1-1Q  |                        | HLA-B*40:01  |           |
| TAWPGAPEV                  | LB-C19ORF48-2E|                     | HLA-B*51:01  |           |
| Peptide          | Cell Line    | HLA    | Source          | Reference |
|------------------|--------------|--------|-----------------|-----------|
| RLRQVGSWL        | LB-FUCA2-1V  | HLA-B*07:02 | GWAS          | (4)       |
| DYLQCVLQI       | ACC-1C       | HLA-A*24:02 | GWAS          | (5)       |
| QLNSVTL         | UTA2-1       | HLA-A*02:01 | GWAS          | (6)       |
| RLHDGRVFV       | LB-TTK-1D    | HLA-A*02:01 | GWAS          | (7)       |
| CVATMIFMI       | UGT2B17/A2   | HLA-A*02:06 | GWAS          |           |
| AEATANGGLAL     | SLCA1A5      | HLA-B*40:02 | GWAS          |           |
| IPRDSWWWVEL      | ZAPHR        | HLA-B*07:02 | GWAS          | (9)       |
| SVLPEDVWW       | DHP1         | HLA-B*57:01 | GWAS          | (10)      |
| WFHHCHPKY       | P2RX7        | HLA-A*29:02 | GWAS          |           |
| STMERWGQKY      | LB-CYBA-1Y   | HLA-A*01:01 | GWAS          |           |
| GISCQGIAV       | LB-DHX33-1C  | HLA-A*02:01 | GWAS          |           |
| KQSPASQLQK      | LB-IMMT-1S   | HLA-A*03:01 | GWAS          | (11)      |
| KYMTAVVKLF      | LB-CYBA-2Y   | HLA-A*23:01/24:02 | GWAS | |
| RPRGARTIQQ      | LB-YIPF1-1T  | HLA-B*07:02 | GWAS          |           |
| HLWSRPRLCL      | LB-STK32C-1R | HLA-B*08:01 | GWAS          |           |
| YYRTNHTVM       | LB-MAN2B1-1T | HLA-C*07:02 | GWAS          |           |
| GQVRLRALY       | LB-GLE1-1V   | HLA-B*15:01 | GWAS          | (12)      |
| GQAGFFPSPF      | LB-ITGB2-1   | HLA-B*15:01 | GWAS          |           |
| AVHNGLGEGKSQA    | LB-NADK-1K   | HLA-A*03:01 | GWAS          | (13)      |
| SEDLICRL        | LB-NUP133-1R | HLA-B*40:01 | GWAS          | (14)      |
| SETKQRTVL       | LB-SON-1R    | HLA-B*40:01 | GWAS          |           |
| MEQLEQLEL       | LB-SWAP70-1Q | cDNA library |               |           |
| GEPOQDLCTL      | LB-TRIP10-1EPC |               |               |           |
| DYLQYVLQI       | ACC-1Y       | HLA-A*24:02 | genetic linkage | (15)  |
| TPNORQNV      | LRH-1        | HLA-B*07:02 | genetic linkage | (16)  |
| ISKERAEAEL     | HEATR1       | HLA-B*08:01 | genetic linkage | (17)  |
| YIGEVLVSV       | HA-2         | HLA-A*02:01 | peptide elution | (18)  |
| VLHDDLLEA       | HA-1         | HLA-A*02:01 | peptide elution | (19)  |
| RTLDKVLEV       | HA-8         | HLA-A*02:01 | peptide elution | (20)  |
| SVAPALALFPA     | LB-ADIR-1F   | HLA-A*02:01 | peptide elution | (21)  |
| Peptide          | HLA             | Library Type         | Reference(s) |
|------------------|-----------------|----------------------|--------------|
| VTEPGTAQY        | HA-3            | HLA-A*01:01          | (22)         |
| RVWDLPGVLK       | PANEl           | HLA-A*03:01          | (23)         |
| KEFEDDIINW       | ACC-2D          | HLA-B*44:02/03       |              |
| SPSVDKARAE       | SMCY            | HLA-B*07:02          | (24)         |
| IVDCLTEEMY       | DFFRY           | HLA-A*01:01          | (25, 26)     |
| EEKRGSLHVW       | HB-1H           | HLA-B*44:03          | (27)         |
| AELLNIPFLY       | UGT2B17/A29     | HLA-A*29:02          | (28, 29)     |
| AELLNIPFLY       | UGT2B17/B44     | HLA-B*44:02/03       |              |
| RPHAIRRPLAL      | LB-ECGF-1H      | HLA-B*07:02          | (30)         |
| ATLPLLCCAR       | ACC-4           | HLA-A*31:01          | (31)         |
| WATLPLLCCAR      | ACC-5           | HLA-A*33:01          |              |
| MEIFIEVFSHF      | ACC-6           | HLA-B*44:03          | (32)         |
| CIPPDSLFFPA      | C19ORF48        | HLA-A*02:01          | (33)         |
| MAVPPCIGV        | TRIM22          | HLA-A*02:01          | (34)         |
| SRDSRGKPGY       | DBY             | HLA-B*27:05          | (35)         |
| TIRYPDVPY        | RPS4Y           | HLA-B*52:01          | (36)         |
| MQQMRHKEV        | UTY             | HLA-B*52:01          | (37)         |
| EVLLRPGLHFR      | UTY             | HLA-A*33:03          | (38)         |
| RESEEEVSVL       | UTY             | HLA-B*40:01          | (39)         |
| LPHNHTDL         | UTY             | HLA-B*08:01          | (40)         |
### Table S2. T cell clones with identified minor H antigens (n=75)

| HLA allele | associating SNPs | no. of antigens | observed p-value | EC50 (M) |
|------------|------------------|-----------------|------------------|----------|
| A*01:01    | missense SNPs    | 4               | 3.3E-11 - 3.7E-07| 7.0E-09 - 4.1E-06 |
|            | other SNPs       | 1               | 2.9E-14          | 5.9E-08  |
| A*02:01    | missense SNPs    | 7               | 3.4E-21 - 2.9E-04| 1.7E-10 – 7.0E-06 |
|            | other SNPs       | 2               | 2.1E-23 - 7.5E-21| 1.2E-11 - 4.8E-10 |
| A*03:01    | missense SNPs    | 9               | 3.2E-17 - 5.0E-09| 9.0E-11 - 1.0E-06 |
|            | other SNPs       | 0               |                  |          |
| B*07:02    | missense SNPs    | 16              | 1.8E-19 - 3.0E-05| 2.2E-11 - 1.4E-06 |
|            | other SNPs       | 14              | 1.6E-18 - 1.7E-07| 2.2E-10 - 1.5E-06 |
| B*08:01    | missense SNPs    | 6               | 1.5E-12 – 2.7E-07| 6.7E-18 - 6.7E-07 |
|            | other SNPs       | 4               | 2.1E-15 - 9.1E-06| 4.6E-08 - 1.8E-06 |
| C*07:01    | missense SNPs    | 2               | 1.4E-14 - 1.2E-08| 8.4E-09 - 1.1E-08 |
|            | other SNPs       | 0               |                  |          |
| C*07:02    | missense SNPs    | 2               | 3.0E-11 - 5.3E-10| 3.1E-08 - 2.4E-04 |
|            | other SNPs       | 0               |                  |          |
| Other      | missense SNPs    | 7               | 2.3E-09 - 5.1E-04| 1.1E-10 - 3.2E-06 |
|            | other SNPs       | 1               | 2.4E-08          | 2.63E-06 |

### Table S3. T cell clones with yet unidentified minor H antigens (n=8)

| HLA allele | associating SNPs | no. of antigens | observed p-value |
|------------|------------------|-----------------|------------------|
| A*02:01    | missense SNP     | 1               | 5.04E-15         |
| A*03:01    | missense SNP     | 1               | 2.53E-11         |
| B*07:02    | missense SNPs    | 3               | 1.36E-16 - 2.32E-11 |
|            | other SNP        | 1               | 4.88E-11         |
| B*08:01    | other SNP        | 1               | 7.23E-07         |
| B*44:02    | missense SNP     | 1               | 5.46E-08         |
| B*44:03    |                  |                 |                  |
SI Methods

Summary of the methodology

The methodology used for the minor H antigen identifications is exactly according our recently published manuscript (11) which is schematically depicted in Figure 2D. In short, 83 T cell clones exclusively recognizing patient-derived, but not donor EBV-transformed B cells (EBV-LCLs) were tested for reactivity against a panel of 191 EBV-LCLs of the 1000 Genomes Project. The IFN-γ secretion profile of each T cell clone against the individual EBV-LCLs in the panel was first analyzed to determine the HLA restriction of the minor H antigens (examples in SI Appendix, Figure S1, overview in SI Appendix, Tables S2 and S3). The data of restricted HLA allele positive individuals was subsequently used to run a Whole Genome Association Scanning (GWAS) analysis to identify SNPs correlating with the recognition pattern of these EBV-LCLs (SI Appendix, Figure S1).

The majority of associations were highly significant on a genome-wide scale (p < 5 * 10^-8) (SI Appendix, Tables S2 and S3). A large proportion of these most significant SNPs were missense SNPs (for 61 of the 83 T cell clones), suggesting that its encoded polymorphic peptide sequence may be the sought minor H antigen recognized by the respective T cell clone (SI Appendix, Tables S2 and S3). For each of the SNPs, various synthetic peptides covering the SNP were loaded on minor H antigen negative but restricted HLA allele positive EBV-LCLs (11). These cells were then evaluated for their capacity to activate the respective T cell clones. 75 of these epitopes were recognized by the respective T cell clones, after which the EC50 was determined (SI Appendix, Table S2). As described in the manuscript, 8 of the 83 minor H antigens were not elucidated using this methodology of which 6 were associated with nonsense SNPs (SI Appendix, Table S3).

Patients

Samples were collected from patients after approval by the LUMC Institutional Review Board and obtaining written informed consent according to the Declaration of Helsinki. Samples were de-identified prior to use in our study. The patients underwent allogeneic stem cell transplantation as treatment for their hematological malignancy. T cell clones were derived from peripheral blood cells and cultured in IMDM supplemented with fetal bovine serum (5%), human serum (5%, Sanquin) and IL-2 (120 IU/ml, Novartis) using a standard PHA (Remel) and feeder stimulation protocol as described in detail before (11).

EBV-LCL culture and T cell reactivity assays

EBV-LCLs (Coriell Cell Repositories) were cultured in IMDM with 10% fetal bovine serum. Cells were seeded in duplicate in 96-well plates and cryopreserved. T cell clones were tested for recognition of EBV-LCLs by overnight incubation T cells with freshly thawed EBV-LCLs loaded with or without peptide in the presence of IL-2 (20 IU/ml) in 384-well plates. Culture supernatant was analyzed for the presence of IFN-γ by ELISA according to manufacturer’s instructions (Sanquin). 83 T cell clones exclusively recognizing patient-derived, but not donor-derived EBV-LCLs were selected for epitope identification based on whole genome association scanning (GWAS).

Whole Genome Association Scanning

For each T cell clone, HLA restriction was determined by analyzing the shared expression of a single HLA class I molecule by EBV-LCLs that were positively recognized in the reactivity assay. For the GWAS, all EBV-LCLs expressing the restricted HLA class I allele were included, while the other EBV-LCLs were excluded. The alleles of all genotyped SNPs in the EBV-LCLs were associated with T cell recognition patterns of the same EBV-LCLs by a Fisher’s exact test using Plink. SNPs with a p-value cut-off at 10^-5
were selected for epitope validation experiments. Gene regions surrounding the associating SNP were in silico translated in 6 frames and analyzed for peptides with predicted binding to the restricted HLA allele by NetMHCpan 4.1. Data was visualized using 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 and to calculate EC50 values, stem cell donor-derived 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.
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