Correction

**IMMUNOLOGY AND INFLAMMATION**


The authors note that Figs. 4 and 5 appeared incorrectly. The errors pertain only to the depiction of the exemplary ELISPOT wells and the UPNs and do not affect the quantitative results displayed in the bar graphs nor any of the results stated and discussed in the paper. The corrected figures and their legends appear below.
### Table

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<th>Peptide</th>
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### Fig. 4

LiTAs are specifically recognized by CLL patient immune responses. (A) HLA class I LiTAs and corresponding LiTAPs (3 HLA-A*03, 5 HLA-A*02, 6 HLA-B*07) functionally evaluated in IFNγ ELISPOT assays. Absolute numbers and frequencies of peptide-specific immune recognition by CLL patient PBMC are summarized in the right hand column. (B) Example of A*03 LiTAPs evaluated in ELISPOT using HV PBMC as a control. An EBV epitope mix containing five frequently recognized peptides [BRLF109–117 YVLDHLIVV (A*02), EBNA3471–479 RLRAEAQVK (A*03), EBNA3247–255 RPPIFIRRL (B*07), BZLF1190–197 RAKFKQLL (B*08), EBNA6162–171 AEGGVGWHRW (B*44)] was used as positive control, HIV GAG18–26 A*03 peptide KIRLRPGGK served as negative control. (C) Example of ELISPOT assays using HLA-A*03 LiTAPs (n = 3) on PBMC of three different CLL patients. Results are shown for immunoreactive LiTAPs. EBV epitope mix served as positive control, HIV GAG18–26 A*03 peptide as negative control. (D) Example of HLA-A*03 benign tissue-derived LiBAPs (n = 3) tested on CLL patient PBMC as internal control for the target selection strategy. EBV epitope mix served as positive control, HIV GAG18–26 A*03 peptide as negative control. (E) Scatterplot of the allele-adjusted frequencies of LiTAP presentation in CLL ligandomes (as detected by MS) and the corresponding allele-adjusted frequencies of immune recognition by CLL patient PBMC in IFNγ ELISPOT. Data points are shown for the 14 of 15 LiTAPs showing immune recognition. HV, healthy volunteer; LiBAP, ligandome-defined benign tissue-associated peptide; LiTAP, ligandome-defined tumor-associated peptide; MS, mass spectrometry; neg., negative; pos., positive; UPN, uniform patient number.
Fig. 5. Identification of additional/synergistic HLA class II LiTAA and LiTAPs. (A) Overlap of HLA class II ligand source proteins of primary CLL samples (n = 20) and HV PBMC (n = 13). (B) Comparative profiling of HLA class II ligand source proteins based on the frequency of HLA restricted representation in CLL and HV PBMC ligandomes. Frequencies [%] of CLL patients/HVs positive for HLA restricted presentation of the respective source protein (x axis) are indicated on the y axis. The box on the left highlights the subset of source proteins showing CLL-exclusive representation in >20% of patients (LiTAA, ligandome-defined tumor-associated antigens). (C) HLA class II LiTAA and corresponding LiTAPs (n = 6) functionally evaluated in IFN-γ ELISPOT assays. Absolute numbers and frequencies of peptide-specific immune recognition by CLL patient PBMC are summarized in the right column. (D) Example of HLA class II LiTAPs evaluated in ELISPOT using HV PBMC as a control. PHA was used as positive control. FLNA_1669-1683 HLA-DR peptide (ETVITVDTKAAKGK) served as negative control. (E) Example of ELISPOT assays using HLA class II LiTAPs (n = 6) on PBMC of three different CLL patients. Results are shown for immunoreactive LiTAPs. PHA was used as positive control, FLNA_1669-1683 HLA-DR peptide served as negative control. (F) Overlap analysis of CLL-exclusive HLA class I and HLA class II ligand source proteins for shared/synergistic vaccine targets. (G) Heatmap analysis of the 132 shared HLA class II LiTAA identified in D. The two source proteins showing representation in ≥20% of both, HLA class I and II CLL patient ligandomes are specified.
HLA ligandome analysis identifies the underlying specificities of spontaneous antileukemia immune responses in chronic lymphocytic leukemia (CLL)


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The breakthrough development of clinically effective immune checkpoint inhibitors illustrates the potential of T-cell–based immunotherapy to effectively treat malignancies. A remaining challenge is to increase and guide the specificities of anticancer immune responses, e.g., by therapeutic vaccine or by adoptive T-cell transfer. By analyzing the landscape of naturally presented HLA class I and II ligands of primary chronic lymphocytic leukemia (CLL), we delineated a novel category of tumor-associated T-cell antigens based on their exclusive and frequent representation in the HLA ligandome of leukemic cells. These antigens were validated across different stages and mutational subtypes of CLL and found to be robustly represented in HLA ligandomes of patients undergoing standard chemotherapy. We demonstrate specific immune recognition of these antigens exclusively in CLL patients, with the frequencies of representation in CLL ligandomes correlating with the frequencies of immune recognition by patient T cells. Moreover, retrospective survival analysis revealed survival benefits for patients displaying immune responses to these antigens. These results directly imply these nonmutant self-peptides as pathophysiologically relevant tumor antigens and encourage their implementation for cancer immunotherapy.

Results

Primary CLL Cells Display No Loss or Down-Regulation of HLA Expression. HLA loss or down-regulation in malignancies may pose a major limitation for T-cell–based immunotherapy (17). Therefore, we determined the HLA expression levels on primary CD19+CD5− CLL cells compared with autologous CD19+CD5− B lymphocytes. HLA surface levels were quantified by flow cytometry in a panel of 7 CLL patients, which revealed patient-specific immune responses, e.g., by therapeutic vaccination or by adoptive T-cell transfer. By analyzing the landscape of naturally presented CLL antigens and identifying the underlying mechanism of tumor control by the immune system. In this study, we directly analyzed the landscape of naturally presented CLL antigens and identified targets conveying immunity against disease. These novel antigens might be useful both for patient stratification and for inducing therapeutic antitumor immunity. Taken together, we demonstrate that CLL is subject to spontaneous T-cell responses targeting nonmutated antigens, which are associated with improved patient survival and provide novel options for the immunotherapy of CLL.

Significance

Effective cancer immunotherapy relies on specific immune recognition of tumor-associated and tumor-specific antigens. In chronic lymphocytic leukemia (CLL), the highly variable course of disease implicates an underlying mechanism of tumor control by the immune system. In this study, we directly analyzed the landscape of naturally presented CLL antigens and identified targets conveying immunity against disease. These novel antigens might be valuable both for patient stratification and for inducing therapeutic antitumor immunity. Taken together, we demonstrate that CLL is subject to spontaneous T-cell responses targeting nonmutated antigens, which are associated with improved patient survival and provide novel options for the immunotherapy of CLL.


The authors declare no conflict of interest.

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individual heterogeneity with total HLA class I molecule counts ranging from ~42,500–288,500 molecules/cell on CLL cells and ~32,000–256,500 molecules per cell on normal B cells. Patient-individual analysis of HLA surface expression in triplicates revealed small, albeit significant differences in expression levels \((P < 0.01)\) for 4 of 7 patients (Fig. 1A). HLA-DR expression ranged from ~29,000–100,500 on CLL cells and ~19,500–79,500 on normal B cells. Minor differences in HLA-DR levels \((P < 0.01)\) were detected for 5 of 7 patients. Statistical analysis of mean surface expression of HLA class I and II on CLL cells compared with normal B cells showed no significant differences (Fig. 1 C and D).

**Mass Spectrometry (LC-MS/MS) Identifies a Vast Array of Naturally Presented HLA Class I and II Ligands.** Mapping the HLA class I ligandomes of 30 CLL patients, we were able to identify a total of 18,844 different peptides representing 7,377 source proteins (Table S1), attaining \(>95\%\) of maximum attainable coverage (Fig. S1). The numbers of different peptides identified per patient ranged from 345 to 2,497 (mean 1,131). Overall, peptides restricted by more than 30 different HLA-A and -B alleles (covering \(>99\%\) of the Caucasian population; calculated according to ref. 18) were identified in this study. In the healthy volunteer (HV) cohort of 30 PBMC donors, a total of 17,322 unique peptides representing 7,180 different source proteins were identified (\(>90\%\) coverage, Table S2). The HLA allotype distribution in the HV cohort covered \(100\%\) of HLA-A and \(>80\%\) of HLA-B alleles in the CLL patient cohort.

Analysis of the HLA class II ligandomes was performed for 20 CLL patients. A total of 5,059 unique peptides representing 1,486 source proteins was identified (Table S1). The HLA class II HV cohort of 13 PBMC donors yielded 2,046 different peptides representing 756 source proteins (Table S2).

**Comparative Profiling of HLA Class I Ligandomes Reveals a Distinct Signature of CLL-Associated Antigens.** To identify novel CLL-associated antigens, we compared the HLA ligand source proteomes of the CLL and HV cohorts. Overlaps of HLA source proteins revealed 2,148 proteins (29.1\% of the mapped CLL source proteome) to be exclusively represented in the HLA ligandome of CLL (Fig. 2C). With the aim of designing a broadly applicable off-the-shelf peptide vaccine, we subsequently prioritized the selection of potential targets according to the following criteria: CLL-exclusivity was defined as paramount criterion, followed by ranking of antigens according to frequency of representation in CLL ligandomes (Fig. 2B). Our platform highlighted 49 source proteins (0.7\% of the CLL source proteome) represented by 225 different HLA ligands showing CLL-exclusive representation in \(\geq20\%\) of CLL patients (Table S3). Applying the same antigen ranking strategy to HV PBMC exclusive antigens, a set of 70 ligandome-defined benign tissue-associated antigens (LiBAAs) and 273 corresponding ligands (LiBAPs) were identified (Table S4) for use as internal control in immunological assays. To estimate the false discovery rate of LiTAA identifications, randomized virtual ligandomes were simulated in silico and the resultant numbers of false positive LiTAAAs were calculated and plotted for different frequencies of representation (Fig. 2D). For the implemented threshold of \(\geq20\%\) CLL-exclusive antigen presentation the false discovery rate was estimated to be 15.3\%.

To extrapolate the number of original LiTAAAs yielded at increased cohort sizes we randomly sampled size-reduced cohorts and defined the resultant LiTAAAs (for each whole-numbered fraction). Nonlinear expression analysis locates the plateau value of LiTAAAs at 69.7 proteins (Fig. 2C).

Apart from broadly represented CLL-LiTAAAs suited for the design of off-the-shelf vaccines, a second panel of 2,099 CLL-exclusive antigens with representation frequencies \(<20\%\) was identified. These targets lend themselves as repositories for more individualized therapeutic approaches (19).

**HLA Class I LiTAAAs Are Derived from Diverse Pathways and Do Not Exhibit Clear Unifying Characteristics Other Than Tumor-Associated Presentation.** Functional annotation clustering of HLA class I LiTAAAs with respect to their biological processes (GO term BP analysis) using DAVID (20) yielded several small, slightly-enriched clusters of proteins involved in nucleotide metabolism, lymphocyte activation, cell migration, transcriptional regulation and apoptosis (Table S5). These clusters were found to be distinct from functional clusters found in HLA class I LiBAAs and HLA class I common “housekeeping” source proteins. However, the

**Fig. 1.** HLA surface expression of primary CLL samples. HLA class I (A) and HLA class II (B) expression on CD5+CD19+ CLL cells compared with autologous CD5−CD19− B cells in 7 primary CLL samples. Data are expressed as mean ± SD of triplicate experiments. (C) Mean HLA class I and (D) HLA class II expression on CD5+CD19+ CLL cells compared with autologous CD5−CD19− B cells \((n = 7)\). *\(P < 0.01\). UPN, uniform patient number.
small sizes and low enrichment scores of these clusters do not indicate LiTAA to be associated with specific, characteristic biological processes. KEGG pathway analysis (21) of HLA class I LiTAA did not identify any overrepresented pathways. To assess whether LiTAA show CLL-associated overexpression on the mRNA level, we investigated a publicly available dataset (GEO profiles, dataset GDS3902), which contains gene expression profiles of 12 CLL patient peripheral blood samples compared with peripheral blood from 5 healthy volunteers (22). We found 11 of the 49 LiTAA to be overexpressed in these CLL patients, 5 of 49 LiTAA to be down-regulated, and 33 of 49 LiTAA not to show any significant differences in gene expression (Table S6).

Fig. 2. Identification of a novel category of tumor-associated antigens by HLA ligandome profiling. (A) Overlap of HLA class I ligand source proteins of primary CLL samples (n = 30) and HV PBMC (n = 30). (B) Comparative profiling of HLA class I ligand source proteins based on the frequency of HLA restricted representation in CLL and HV PBMC ligandomes. Frequencies [%] of CLL patients/HVs positive for HLA restricted presentation of the respective source protein (x axis) are indicated on the y axis. The box on the left highlights the subset of source proteins showing CLL-exclusive representation in >20% of patients (LiTAA, ligandome-defined tumor-associated antigens). (C) Regression analysis of the number of LiTAA yielded at different cohort sizes. Reduced-size CLL and HV PBMC cohorts were randomly assembled from the complete respective cohorts (n = 30) and LiTAA were defined as CLL-exclusive proteins presented on ≥20% of CLL patients. The process of random cohort assembly and LiTAA definition was repeated 100 times and the resultant mean number of LiTAA was plotted for cohort sizes of n = 5, 10, 15, 20 and 25. Regression analysis using an exponential model extrapolated the plateau value for the number of LiTAA resulting at high values of n to be 69.7. (D) Statistical analysis of the proportion of false positive LiTAA identifications at different representation frequencies. The numbers of original LiTAA identified based on the analysis of the CLL and HV PBMC cohorts were compared with random virtual LiTAA. Virtual CLL patients and HV PBMC were generated in silico based on random weighted sampling from the entirety of protein identifications in both original cohorts. These randomized virtual ligandomes of defined size (n = 1130 proteins, which is the mean number of protein identifications in all analyzed samples) were used to define LiTAA based on simulated cohorts of 30 CLL versus 30 HV PBMC. The process of protein randomization, cohort assembly and LiTAA identification was repeated 1,000 times and the mean value of resultant virtual LiTAA was calculated and plotted for the different threshold values. The corresponding false discovery rates for any chosen LiTAA threshold are listed below the x axis. (E) Representation of published CLL-associated antigens in HLA class I ligandomes. Bars indicate relative representation [%] of respective antigens by HLA class I ligands on CLL and HV PBMC. Dashed lines divide the antigens into three groups according to their degree of CLL-association (CLL exclusive, CLL overrepresented, not overrepresented).
Detection of Naturally Presented HLA Class I Ligands Derived from Established CLL-Associated Antigens. Alongside the identification of novel CLL-associated antigens, a secondary approach focused on the ranking of the few established CLL-antigens within our dataset. We identified 28 different HLA ligands representing 8 described CLL-associated antigens (Table S7). Notably, only fibromodulin (FMOD\textsubscript{234-333}, RINEFSISSE, HLA-B*15) showed CLL-exclusive representation, ranking at #437 of CLL antigens in our dataset, due to low frequency of representation in the CLL patient cohort. The remaining 7 antigens showed representation both on CLL and HV PBMC, thus failing to fulfill the criterion of CLL-exclusivity. However, for CD19, CD20, RHAMM and PRAME, CLL-associated overrepresentation of varying degrees was detected (Fig. 2E).

Comparative Ligandome Profiling Identifies LiTAA\textsubscript{s} Shared Among Different Disease Stages and Risk Strata. To assess the applicability of the novel targets across different stages of disease, we performed subset-specific ligandome profiling comparing patients in disease stages Binet A (n = 9), B (n = 7), and C (n = 14). Overlap analysis of the 2,148 CLL-exclusive source proteins revealed that 550 (25.6\%) of them were shared among at least two stages, with a core group of 137 proteins (6.1\%) represented in patients of all three disease stages (Fig. 3A). Of note, 45 of 49 (91.8\%) of LiTAA\textsubscript{s} (Table S3) belong to the core group of shared source proteins represented in all three subsets. Heatmap analysis of the representation frequencies of all 49 LiTAA\textsubscript{s} across Binet stages A, B, and C is shown in Fig. 3B.

Another focus was placed on determining the representation of LiTAA\textsubscript{s} in the subsets of high-risk patients carrying the 17p13 deletion (del17p, n = 5) compared with patients without this genetic aberration (no del17p, n = 25). We found 77.7\% of the identified LiTAA\textsubscript{s} to be represented in both subsets (Fig. 3C). Together, these data support the devised strategy of cohort-comprising analysis of HLA ligandome for selection of broadly applicable targets.

Functional Characterization of HLA Class I LiTAA\textsubscript{s} Reveals CLL-Associated Immunoreactivity. To evaluate the immunogenicity and specificity of our HLA class I LiTAA\textsubscript{s}, we performed 12-d recall IFN\textsubscript{γ} ELISPOT assays. A panel of 15 LiTAA\textsubscript{s} (6 A*02, 3 A*03, and 6 B*07 LiTAA\textsubscript{s}) was implemented for stimulation of HLA-matched PBMC obtained from CLL patients and healthy volunteers (Fig. 4). We observed IFN\textsubscript{γ} secretion for 14 of 15 (93.3\%) of tested LiTAA\textsubscript{s} in CLL patients (Fig. 4A and C and Fig. S2 C and F), but not in healthy controls (0 of 10, Fig. 4B and Fig. S2 B and E). These findings were confirmed exemplarily for PA\textsubscript{A*03} (DMXL1\textsubscript{1271-1279} SSSGLHPPK) by tetramer staining of CD8\textsuperscript{+} T cells and intracellular cytokine staining for IFN\textsubscript{γ} and TNF\textalpha (Fig. S3 A and B). ELISPOT assays using HLA-matched benign tissue-derived LiBAA\textsubscript{s} were performed to control for the CLL-specificity of the observed LiTAA-directed immune recognition in CLL patients. We tested a panel of 9 LiBAA\textsubscript{s} (3 A*02, 3 A*03, 3 B*07, Table S4) and observed no significant IFN\textsubscript{γ} secretion in any of the tested CLL patients [0 of 7 A*03 (Fig. 3D), 0 of 10 A*02 and 0 of 5 B*07 (Fig. S2 A and D)].

The Frequencies of HLA Restricted Presentation and Immune Recognition Show a Direct Correlation. For the 14 of 15 LiTAA\textsubscript{s} showing immune recognition in one or more patients, we calculated the allele-adjusted frequencies of HLA restricted presentation (as detected by LC-MS/MS) and the frequencies of immunoreactivity (as detected by ELISPOT) in CLL patients. Strikingly, a linear correlation of these two parameters was observed (Pearson’s r = 0.75, R\textsuperscript{2} = 0.56, Fig. 4E). For six patients, we were able to perform both, HLA ligandome analysis and functional characterization in ELISPOT assays. Notably, all three patients that were positive for presentation of specific LiTAA\textsubscript{s} in LC-MS/MS analysis also showed immune recognition of these LiTAA\textsubscript{s} in ELISPOT assays. On the other hand, we did observe immune reactions to LiTAA\textsubscript{s} in five patients, which were negative for the respective peptides in ligandome analysis (Table S8).

HLA Class II Ligandome Analysis Identifies CD4\textsuperscript{+} T-Cell Epitopes for Synergistic Vaccine Design. Because of the important indirect and direct roles of CD4\textsuperscript{+} T cells in anticancer immune responses (23), an optimal vaccine require the inclusion of HLA class II epitopes. We performed overlap analysis of CLL and HV PBMC ligandomes and identified 937 proteins (63.0\% of the identified CLL source proteins) to be exclusively represented in the ligandomes of CLL patients (Fig. 5A). Applying the same antigen-ranking strategy as described for HLA class I, we identified 73 HLA class II LiTAA\textsubscript{s} represented by 460 corresponding LiTAA\textsubscript{s} (Fig. 5B and Table S9). Functional characterization of a panel of 7 HLA class II LiTAA\textsubscript{s} (Fig. 5C) in IFN\textsubscript{γ} ELISPOT assays revealed significant IFN\textsubscript{γ} secretion for 6 of 7 (85.7\%)
LiTAPs in CLL patients (Fig. 5E), but not in healthy controls (0 of 10, Fig. 5D). Next, we performed combined analysis of HLA class I and II ligandomes to identify shared, synergistic targets. Overlap analysis of CLL-exclusive source proteins revealed 132 proteins to be represented both in HLA class I and II ligandomes (Fig. 5F). Heatmap analysis identified 2 proteins displaying representation frequencies ≥20% in both ligandomes [B4GALT1 (26.7% class I/30.0% class II), HLA-DMA (20.0% class I/20.0% class II), Fig. 5G]. Strikingly, one of the class I LiTAPs (HLA-DMA_{206–214} HEIDRYTAI, B*18) was revealed to be completely embedded in the corresponding HLA class II LiTAP (VTHEIDRYTAIAY).

HLA Class I and Class II Ligand Source Proteins Show Distinct Subcellular Distribution Patterns. Bioinformatic analysis based on gene ontology (GO) term clustering implementing DAVID (20) revealed distinct clusters of cellular compartments for HLA ligand source proteins represented in HLA class I and II ligandomes (Table S10). The most distinct clusters identified were a large group of nuclear lumen derived source proteins presented on HLA class I and a highly enriched cluster of vesicle-derived proteins presented on HLA class II. The clusters for HLA class I- and II-shared source proteins generally showed lower levels of enrichment, with the most prominent cluster comprising proteins from the lysosome/endosome pathway, followed by membrane-bound and extracellular proteins.
hinting at cross-presentation on HLA class I as an underlying mechanism. Of note, for HLA class II, two clusters of cell-endogenous source proteins derived from the cytosolic ribosome and the nuclear pore were identified, indicating underlying mechanisms of autophagy and cross-presentation.

**Longitudinal Analysis of CLL Patient Ligandomes Under Different Therapeutic Regimens.** A rational setting to apply peptide-based immunotherapy is maintenance therapy and eradication of MRD. As a consequence, peptide vaccination in CLL would mainly be performed after standard chemo- or immunotherapy. Therefore, we analyzed HLA expression and performed ligandome profiling across different time points of CLL patients undergoing different therapeutic regimens.

We quantified HLA class I and II surface expression in 4 patients undergoing rituximab treatment (Rt0h, Rt24h) and in 1 patient receiving alemtuzumab (At0h, At72h, At7d, Fig. S4 A–D). HLA surface expression showed patient-individual heterogeneity.

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with no significant changes in mean HLA class I ($R_{0h} = 50,500$, $R_{24h} = 48,000$; $A_{0h} = 42,500$, $A_{24h} = 27,500$; $A_{0h} = 47,000$, $A_{7d} = 55,500$) expression over the course of either therapeutic regimen.

Longitudinal HLA class I ligandome profiling was performed in single patients undergoing rituximab-bendamustin, alemtuzumab and ofatumumab treatment, respectively (Fig. 6 A–C). Differential presentation (defined as ≥ twofold change with $P \leq 0.05$ after Bonferroni adjustment for multiple testing) was observed for 0.73% ($n = 14$) of HLA class I ligands under rituximab-bendamustin treatment, for 7.4% ($n = 182$) of ligands under alemtuzumab treatment and for 6.5% ($n = 98$) of ligands under ofatumumab treatment. Overall, 6 LiTAPs representing 6 of the total of 32 LiTAAAs (18.8%) detectable in these three patients were revealed to be differentially presented over the course of therapy. Of note, 5 of 6 (83.3%) of these LiTAPs showed significant up-regulation posttherapy.

**Immunological Responses Against LiTAPs Are Associated with Improved Overall Survival of CLL Patients.** As a last step, we investigated the prognostic relevance of LiTAP-specific immune responses. We performed retrospective survival analysis of 45 CLL patients analyzed by ELISPOT assay (Table S11). We dichotomized patients into groups with T-cell responses specific for 0–1 LiTAPs ($n = 32$) versus >1 LiTAP ($n = 13$) according to previous results in RCC patients demonstrating significantly higher levels of disease control in patients showing responses to multiple antigens (6). We found that 9 of 32 (28.1%) of patients in the low-responding cohort, versus 0 of 13 of patients in the high-responding cohort had died. A strong indication for prolonged overall survival in the high-responding cohort compared with the low responders based on survival time from study enrollment ($P < 0.05$, Fig. 7A) as well as survival time from diagnosis was observed ($P = 0.0695$, Fig. 7B). This analysis does not consider differences in disease stage, cytogenetics or preceding treatments.

**Discussion**
With T-cell–based immunotherapy proclaimed as the scientific breakthrough of 2013 (24), the research field finally revealed its inherent potential for highly effective cancer therapy (25–27). Remaining challenges lie in guiding the specificity and increasing the frequency of antitumor immune responses and expanding the spectrum of targetable entities (26). A rational and promising approach to achieve this goal is (multi) peptide vaccination (28, 29). For this purpose the identification of immunologically relevant, tumor-associated antigens is indispensable and enabled by the direct analysis of naturally presented HLA ligands. Here, we comprehensively mapped the landscape of naturally presented HLA ligands in primary CLL samples and assessed interpatient individuality as well as treatment-induced changes in the HLA ligandome composition. We implemented a new strategy, which defines a novel category of tumor-associated antigens strictly

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**Fig. 6.** Longitudinal HLA class I ligandome analysis of CLL patients undergoing chemo- or immunotherapy. Volcano plots of the relative abundance of HLA ligands in the class I ligandomes of patients after treatment compared with their respective abundance before therapy (ratio post- and pretherapy). Dashed lines indicate the thresholds for differential peptide presentation (defined as ≥ twofold ratio with $P < 0.05$ after Bonferroni correction), with up-regulated ligands in the upper right and down-regulated ligands in the upper left areas. Frequencies and absolute numbers of differentially presented ligands are specified in the respective quadrants. LiTAPs showing differential presentation over the course of therapy are marked in red and their sequences are specified. (A) Analysis of a CLL patient ligandome before therapy, 48 h after treatment with rituximab (375 mg/m$^2$), and 24 h after treatment with bendamustin (90 mg/m$^2$). One of 25 (4.0%) of detectable LiTAPs showed differential presentation above the specified thresholds. (B) Analysis of a CLL patient ligandome before therapy and after the first 7 d of treatment with alemtuzumab (three doses of alemtuzumab, 10 mg, 20 mg, and 30 mg on day 1, 3, and 5; ligandome analysis on day 7). Three of 24 (12.5%) of detectable LiTAPs showed differential presentation above threshold. (C) Analysis of a CLL patient ligandome before therapy and 24 h after treatment with 300 mg of atumumab. Two of 10 (20.0%) of detectable LiTAPs showed differential presentation above threshold.

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based on their exclusive and frequent representation in CLL ligandomes. This approach identified a panel of 49 HLA class I LiTAAs showing broad and frequent representation across different Binet stages and mutational backgrounds as well as robust presentation under chemo-immunotherapy, thus representing suitable candidates for broadly applicable off-the-shelf peptide vaccines, also after standard treatment of CLL. Reverse bioinformatics analysis of LiTAAs, such as functional annotation clustering and gene expression analysis did not lead to the identification of comprehensive unifying characteristics which would enable the categorization or even prediction of LiTAAs. This result indicates the unique character of the HLA ligandome and underscores the importance of defining T-cell antigens by direct differential ligandome profiling. The relatively high false discovery rate (15.3%) at the implemented threshold for LiTAA definition is considered an acceptable tradeoff between assay stringency and discovery rates, as the profiling approach was used as a screening step to identify candidates for subsequent immunological validation.

Recognition of tumor-associated peptides by the immune system is a fundamental requirement for effective T-cell–based immunotherapy (30). Recent studies showing promising results focused on mutated neoantigens, which, in theory, constitute tumor-specific and highly immunogenic targets (23, 31, 32). Here, we observed spontaneous T-cell responses targeting nonmutant epitopes in CLL patients. We validated these immune responses to be CLL-associated and directed strictly against CLL antigens (LiTAPs), thus indicating tumor-dependent priming of T cells specific for nonmutated tumor epitopes in vivo in CLL patients. This immunoreactivity against “self” in the context of cancer has long been established as a component of anticancer immune responses (33) and a range of nonmutant tumor-associated antigens (TAAs) have been described (3). However, recently it became evident that in solid tumors, only a small fraction of the tumor antigens described so far - including mutated and nonmutated epitopes - are frequently and effectively targeted by T-cell responses (34), even in the context of immune checkpoint modulation (35). By corollary, a large proportion of the specificities of tumor-reactive lymphocytes remain undefined. Surprisingly, in the study at hand we fairly frequently observed spontaneous T-cell responses targeting the novel antigen class of LiTAAs in leukemia patients. We also found that some of the established TAAs, when evaluated directly and strictly on the HLA ligandome level, show lacking degrees of tumor association and frequencies of presentation. Similar observations were made in our previous study of AML antigens (36). Together, these results suggest that direct, HLA ligandome-centric approaches might be indispensable for the identification of immunologically relevant, but otherwise inconspicuous, tumor antigens and might help explain the lacking immunogenicity observed for some of the previously proposed TAAs.

Interestingly, for immunoreactive LiTAPs the direct correlation between frequencies of epitope detection in CLL ligandomes and frequencies of LiTAP-specific immune recognition by CLL patient PBMC might indicate that antigen presentation on cancer cells is the major prerequisite/limiting factor for the priming of LiTAP-specific immune responses. The, presence of LiTAP-specific T cells in patients could be explained by the absence of negative thymic selection for these antigens due to tumor-specific alterations in antigen presentation not paralleled in the thymus. Further investigations aiming to elucidate the naïve T-cell repertoire including T-cell receptor affinities and the cellular processes leading to the exclusive appearance of antigen on cancer cells in LiTAP-specific immune responses are ongoing.

As CD4+ T cells play important indirect and direct roles in antitumor immunity (23, 37–42), we further applied our profiling approach to HLA class II ligandomes, identifying a panel of 73 additional class II LiTAAs. Notably, one of the HLA class II LiTAPs was found to contain a complete, embedded HLA class I LiTAP, which points to a striking new option for synergistic vaccine design as discussed in recent publications (43, 44). Such naturally presented embedded HLA ligands might represent optimal vaccine candidates that are recognized by both, CD4+ and CD8+ T cells. Interestingly, the clusters of proteins presented on both HLA class I and II were found to be derived from cellular compartments typically associated with the HLA class II presentation, thus indicating cross-presentation on HLA class I as the mechanistic basis of this dual representation.

To investigate the clinical relevance of anti-LiTAP immune responses in CLL patients, we performed retrospective survival analysis and observed a survival benefit for patients showing immune reactions to multiple of the tested LiTAPs. This result indicates the involvement of these immune responses in disease control in CLL patients and directly implies LiTAAs as pathophysiologically relevant tumor antigens in leukemia. In consequence, these malignancies might be targeted effectively by immunotherapy despite their reported low number of somatic mutations (45), which are currently viewed as the most highly active targets for T-cell–based immunotherapy. The LiTAPs we find on tumor cells based on tumor-specific antigen processing modalities may well be of similar immunogenicity as mutated antigens, if our hypothesis of the absence of processing modalities may well be of similar immunogenicity as mutated antigens, if our hypothesis of the absence of such processing modalities in the thymus is correct.

In conclusion, the HLA ligandome-centric approach might prove an invaluable tool for antigen discovery and re-evaluation as well as for patient stratification. In CLL, it enabled the identification of a panel of novel immunogenic nonmutated epitopes amenable for clinical application and provided a new perspective on the immunogenicity of this malignancy.

Materials and Methods

Patients and Blood Samples. For HLA ligandome analysis, PBMC from CLL patients (>80% CLL cell frequency) as well as PBMC from healthy volunteers...
Peptide samples were separated by chromatography and quantified using LC-MS/MS analysis. Flow cytometric data analysis was performed on a FACSCanto Analyzer (BD).

**Isolation of HLA Ligands from Primary Samples.** HLA class I and II molecules were isolated using standard immunoaffinity purification as described (46, 47) using the pan-HLA class I specific mAb W6/32 and the pan-HLA class II specific mAb TG39, respectively.

**Analysis of HLA Ligands by LC-MS/MS.** Peptide samples were separated by reversed-phase liquid chromatography (nanoUPLC, UltiMate 3000 RSLCnano, Dionex) and subsequently analyzed in an on-line coupled LTQ Orbitrap XL hybrid mass spectrometer (ThermoFisher). Peptides were analyzed in five technical replicates. Sample volumes of 5 μL (sample shares of 20%) were injected onto a 75 μm × 2 cm trapping column (Acclaim PepMap RSLC, Dionex) at 4 μL/min for 5.75 min. Peptide separation was subsequently performed at 50°C and a flow rate of 175 nL/min on a 50 μm × 50 cm separation column (Acclaim PepMap RSLC, Dionex) applying a gradient ranging from 2.4 to 32.0% of ACN over the course of 140 min. Eluting peptides were ionized by nanospray ionization and analyzed in the mass spectrometer implementing a top 5 CID (collision induced dissociation) method generating fragment spectra for the 5 most abundant precursor ions in the survey scans. Resolution was set to 60,000. For HLA class I ligands, the mass range was limited to 400–650 m/z with charge states +2 and +3 selected for fragmentation. For HLA class II, a mass range of 390–1,500 m/z was analyzed with charge states +2 selected for fragmentation.

**Intracellular Cytokine and Tetramer Staining.** The frequency and functionality of peptide-specific CD8+ T cells was analyzed by intracellular IFN-γ and TNF-α staining as described (50, 52). PBMC were pulsed with 10 μg/mL of individual peptide and incubated in the presence of 10 μg/mL Brefeldin A (Sigma Aldrich) and 10 μg/mL GolgiStop (BD Bioscience) for 6–8 h. Cells were labeled using Cytofix/Cytperm (BD), CD8-PECy7 (Beckman Coulter), and IFN-γ-FITC (BD). Samples were analyzed on a FACS Canto II.

The frequency of peptide-specific CD8+ T cells was determined by staining with anti-CD8 and HLA:peptide-tetramer-PE as described (53).

**Database Search and Spectral Annotation.** For data processing, the software Proteome Discoverer (v1.3, ThermoFisher) was used to integrate the search results of the Mascot search engine (Mascot 2.2.04, Matrix Science) against the human proteome as comprised in the Swiss-Prot database (www.uniprot.org/uniprot; release December 27th, 2013; 20,279 reviewed protein sequences contained). The search combined data of technical replicates and was not restricted by enzymatic specificity. Precursor mass tolerance was set to 5 ppm, and fragment mass tolerance was set to 0.5 Da. Oxidized methionine was allowed as a dynamic modification. False discovery rates (FDR) were determined by the Percolator algorithm (48) based on processing against a decoy database consisting of the shuffled target database. FDR was set at a target value of q ≤ 0.05 (5% FDR). Peptide-spectrum matches (PSM) with b ≤ 0.05 were filtered according to additional, orthogonal parameters, to ensure spectral quality and validity. Mascot scores were filtered to ≥20. For HLA class I, peptide lengths were limited to 8–12 aa of length. For HLA class II, peptides were limited to 12–25 aa of length. Protein grouping was disabled, allowing for multiple annotations of peptides (e.g., conserved sequences mapping into multiple proteins). For quality control, yield thresholds of ≥300 unique HLA class I ligands and ≥100 unique HLA class II ligands per sample were applied. HLA annotation was performed using SYFPEITHi (www.syfpeithi.de) or an extended in-house database.

**Longitudinal Analysis of CLL Patient Ligandomes Over the Course of Therapy.** For label-free quantification (LFQ) of the relative HLA ligand abundances over the course of therapy, the injected peptide amounts of paired samples were normalized and LC-MS/MS analysis was performed in five technical replicates for each sample.


