HLA ligandome analysis of primary chronic lymphocytic leukemia (CLL) cells under lenalidomide treatment confirms the suitability of lenalidomide for combination with T-cell-based immunotherapy

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ABSTRACT
Recent studies suggest that CLL is an immunogenic disease, which might be effectively targeted by antigen-specific T-cell-based immunotherapy. However, CLL is associated with a profound immune defect, which might represent a critical limitation for mounting clinically effective antitumor immune responses. As several studies have demonstrated that lenalidomide can reinforce effector T-cell responses in CLL, the combination of T-cell-based immunotherapy with the immunomodulatory drug lenalidomide represents a promising approach to overcome the immunosuppressive state in CLL. Antigen-specific immunotherapy also requires the robust presentation of tumor-associated HLA-presented antigens on target cells. We thus performed a longitudinal study of the effect of lenalidomide on the HLA ligandome of primary CLL cells in vitro. We showed that lenalidomide exposure does not affect absolute HLA class I and II surface expression levels on primary CLL cells. Importantly, semi-quantitative mass spectrometric analyses of the HLA peptidome of three CLL patient samples found only minor qualitative and quantitative effects of lenalidomide on HLA class I- and II-restricted peptide presentation. Furthermore, we confirmed stable presentation of previously described CLL-associated antigens under lenalidomide treatment. Strikingly, among the few HLA ligands showing significant modulation under lenalidomide treatment, we identified upregulated IKZF-derived peptides, which may represent a direct reflection of the cereblon-mediated effect of lenalidomide on CLL cells. Since we could not observe any relevant influence of lenalidomide on the established CLL-associated antigen targets of anticancer T-cell responses, this study validates the suitability of lenalidomide for the combination with antigen-specific T-cell-based immunotherapies.

Introduction
In recent years T-cell-based immunotherapy has become a main pillar of anticancer therapy.\textsuperscript{1-9} However, profound immune defects in some cancer entities and immune escape mechanisms represent major limitations of these immunotherapeutic approaches.\textsuperscript{10,11} To overcome this obstacle and thereby to increase overall response rates in cancer patients, the combination of T-cell-based therapies with immunomodulatory drugs seems indispensable.

Chronic lymphocytic leukemia (CLL) represents a B-cell malignancy that shows characteristics of immunogenicity and immunosuppression: The immunogenicity, which is documented by graft versus leukemia effects after haematopoietic stem cell transplantation,\textsuperscript{12,13} and by cases of spontaneous remissions after viral infections,\textsuperscript{14} as well as favorable immune effector-to-target cell ratios in the minimal residual disease setting, suggest that CLL might be effectively targeted by T-cell-based immunotherapy.\textsuperscript{15} On the other hand, CLL is associated with profound immune defects, characterized by defective CD8\textsuperscript{+} and CD4\textsuperscript{+} T-cell function,\textsuperscript{16} increased frequencies of regulatory T cells\textsuperscript{17} and defective immunological synapse formation,\textsuperscript{18} which result in increased susceptibility to recurrent infections as well as failure to mount effective antitumor immune responses. Lenalidomide, an immunomodulatory compound targeting both cancer cells and their microenvironment, has shown substantial activity in several hematological malignancies.\textsuperscript{19} It has been approved for the treatment of multiple myeloma, myelodysplastic syndrome and mantle cell lymphoma and is currently investigated for the treatment of CLL.\textsuperscript{20,21} Several preclinical and clinical studies have proven the
positive immunomodulatory effect of lenalidomide treatment on T-cell responses in CLL, demonstrating enhanced antigen uptake and improved priming of CD8\(^+\) T cells by antigen-presenting cells,\(^{22}\) reduction of regulatory T cells,\(^{23}\) increased frequency of functional CD8\(^+\) and CD4\(^+\) T cells,\(^{24}\) upregulation of costimulatory molecules on CLL cells\(^{25}\) as well as the reconstitution of the defective T-cell immune synapse.\(^{18,26}\) Therefore, lenalidomide can be considered well suited for combination with antigen-specific T-cell-based immunotherapeutic approaches in CLL. We recently conducted a study characterizing the antigenic landscape of CLL by mass spectrometric analysis of naturally presented HLA ligands and identified a panel of CLL-specific CD8\(^+\) and CD4\(^+\) T-cell epitope targets suitable for T-cell-based immunotherapy approaches in CLL patients.\(^{27}\) Anticancer drugs can have marked effects on the HLA ligandome of tumor cells,\(^{28,29}\) including changes in HLA surface expression,\(^{30,31}\) the HLA allotype distribution,\(^{32}\) as well as the induction of novel treatment-associated ligands.\(^{33}\) For the combination of T-cell-based immunotherapy with other anticancer drugs it is thus of great importance to characterize the effects of these drugs not only on the effector cells but also on the antigenic landscape of the target cells. For lenalidomide, it was recently shown that its clinical activity in CLL is not only mediated via the microenvironment but also by direct inhibition of CLL cell proliferation via cereblon.\(^{34}\) This leads to the upregulation of the cyclin-dependent kinase inhibitor p21WAF1/Cip1 and to the increased degradation of the transcription factors IKZF1 and IKZF3.\(^{34}\) These direct effects of lenalidomide might influence HLA ligand presentation of CLL cells. In the present study, we therefore comprehensively and semi-quantitatively mapped the impact of lenalidomide on HLA-restricted antigen presentation in primary CLL samples.

## Results

### In vitro lenalidomide has no significant impact on HLA surface expression of primary CLL cells

To assess the impact of lenalidomide on HLA surface expression, we performed longitudinal quantification of HLA class I and II surface molecule counts on primary CLL cells as well as autologous B cells of four patients upon in vitro incubation with lenalidomide. First, we analyzed the cytotoxicity of treatment with low dose (0.5 \(\mu\)M) lenalidomide on primary CLL samples. Viability analyses showed no significant difference between lenalidomide-treated cells and untreated controls, with mean cell viability of 71% and 74% at \(t_{48h}\), respectively. With regard to HLA class I expression on CD19\(^-\)CD5\(^-\)CLL cells, no significant impact of lenalidomide compared with untreated controls was observed (fold-change 0.92–1.02, \(t_{48h}\)), with expression levels ranging from 60,000–125,000 molecules/cell (Fig. 1A and B). For HLA class II, a slight increase of HLA surface molecules after lenalidomide treatment was detectable (fold-change 1.25–1.43, \(t_{48h}\)) with expression levels ranging from 29,000 to 201,000 molecules/cell (Figs. 1C and D). In line, HLA class I and II quantification of autologous CD19\(^-\)CD5\(^+\) B cells showed no significant impact of in vitro lenalidomide exposure compared with untreated controls (Fig. S1).

![Figure 1](image-url)

**Figure 1.** Effect of in vitro lenalidomide treatment on HLA class I and II surface expression on primary CLL cells. Quantification of HLA surface expression was performed using a bead-based flow cytometric assay using the pan-HLA class I-specific monoclonal antibody W6/32 and the HLA-DR-specific monoclonal antibody L243. Absolute counts of HLA class I (A) and HLA class II (C) surface molecules on primary CD19\(^-\)CD5\(^+\) CLL cells (n = 4) treated in vitro with lenalidomide. Longitudinal analysis of relative changes (normalized to untreated controls) in HLA class I (B) and HLA class II (D) surface expression on primary CLL cells under in vitro lenalidomide treatment. Abbreviations: ns, not significant (\(p \geq 0.05\), unpaired \(t\)-tests); UPN, uniform patient number.
Relative quantitation of HLA class I peptide presentation on primary CLL cells under in vitro lenalidomide treatment

To assess changes in HLA class I ligandome composition, direct mass spectrometric analysis of HLA class I ligand extracts was performed for three primary CLL samples before treatment and at \( t_{24h} \) and \( t_{48h} \), as well as for the corresponding untreated controls. Based on available PBMC counts (Fig. 2A), we performed in vitro lenalidomide treatment of UPN1 in three biological replicates and for UPN2 and UPN3 in single experiments. In total, 6,991 uniquely presented HLA class I ligands representing 3,983 source proteins were identified on these primary CLL cells (\( n = 3 \), Fig. 2A, Supplemental Data 1–3). Within this data set, we were able to detect 35 different HLA-matched CLL-associated ligands (UPN1, 27; UPN2, 5; UPN3, 4) described in a previous study by our group (Figs. 2A and B).27 Using the summed peptide intensities of all FDR-filtered HLA ligand identifications as an indirect measure of total peptide abundance, we did not detect any major decrease of total HLA class I peptide presentation on lenalidomide-treated cells (\( t_{24h} \) and \( t_{48h} \) combined) compared with levels before treatment or untreated controls (UPN1, +35.1%; UPN2, –11.4%; UPN3, +3.1%; Fig. 2C, Figs. S4A and D).

Lenalidomide has no substantial influence on the relative abundance of HLA-presented peptides and does not induce cryptic, treatment-associated HLA ligands

A first basic qualitative comparison of the HLA peptidomes of untreated versus lenalidomide-treated primary CLL cells using overlap analyses of HLA ligand identifications based on high-quality peptide spectrum matches filtered for 5% FDR and HLA-binding affinity suggests considerable differences in HLA ligandome composition. Comparison of the untreated samples (UPN1, 2 and 3 cells before treatment, and at \( t_{24h} \) and \( t_{48h} \) without lenalidomide exposure) with the treated samples revealed 8.3% (382/4,603 HLA ligands), 43.7% (469/1,073 HLA ligands) and 30.9% (529/1,710 HLA ligands) of the HLA ligandomes of UPN1, 2 and 3 to be exclusively presented on untreated cells, respectively. Whereas 22.3% (1,026/4,603 HLA ligands, UPN1), 4.8% (51/1,073 HLA ligands, UPN2) and 12.3% (210/1,710 HLA ligands, UPN3) were exclusively presented on lenalidomide-treated cells.

Figure 2. Mass spectrometric analysis of the HLA class I-presented peptidome of primary CLL cells under in vitro lenalidomide treatment. (A) Overview of PBMC count, unique HLA class I ligand IDs, representing source protein IDs and the number of identified HLA-matched CLL-associated antigens identified by mass spectrometry of analyzed primary CLL samples (\( n = 3 \)). (B) Overlap analysis of HLA class I ligands identified on UPN1 with HLA-matched CLL-associated class I antigens identified in an earlier study. (C) HLA class I ligand extracts of UPN1 before in vitro treatment and at \( t_{24h} \) and \( t_{48h} \) after incubation with 0.5 \( \mu \)M lenalidomide or 0.005% DMSO (vehicle control) were analyzed in biological triplicates. The number of HLA ligand identifications and the summed area of their extracted ion chromatograms are indicated in gray and black bars, respectively. The threshold for relative quantitation (TRQ) was set to 500 HLA ligand IDs. Abbreviations: UPN, uniform patient number; TRQ, threshold for relative quantitation.
HLA ligands, UPN3) of the HLA ligandomes were only detectable after treatment with lenalidomide (Fig. 3A, Figs. S4B and E). Out of the 1,287 treatment-exclusive HLA ligands, 284 (22%) were never identified on any benign or malignant tissue comprised in our in-house database containing 260 HLA ligandomes of various normal tissues and organ specimens as well as 262 ligandomes of different malignant entities. To asse if any of these treatment-exclusive HLA ligands are significantly associated with lenalidomide treatment, we plotted the frequencies of peptide detection in the two different conditions (lenalidomide-treated n = 6, untreated n = 8, UPN1) and calculated the significance thresholds for treatment-associated presentation of HLA ligands based on permutation analysis as described previously (Figs. 3B and S8A). Notably, none of the 1,026 UPN1 HLA ligands exclusively detected on treated cells was found to reach the thresholds for significant association with lenalidomide treatment (p < 0.05).

Due to the fact that the missing value problem is a major concern in data-dependent acquisition mass spectrometry (DDA MS) and label-free quantitative proteomics, we further used the strategy of matching between runs which reduces missing values in quantitation by lowering FDR cutoffs and

![Figure 3](image-url)

**Figure 3.** Quantitative and qualitative influence of in vitro lenalidomide treatment on the HLA class I peptidome of UPN1. (A) Overlap analysis of HLA class I ligands identified on lenalidomide- vs. untreated (vehicle controls and pre treatment) cells. (B) Frequency-based analysis of peptide presentation on treated vs. untreated (vehicle controls and pre treatment) UPN1 cells. HLA class I ligands are indicated on the x-axis, the frequency of positive ligandomes on the y-axis. (C–F) Volcano plots of the relative abundances of HLA ligands on UPN1 cells comparing the conditions indicated combining three biological replicates. Each dot represents a specific HLA ligand. Log2 fold-changes of peptide abundance are indicated on the x-axis, the corresponding significance levels after multi-testing correction (−log10 p-value) on the y-axis. HLA ligands showing significant up or downmodulation (≥ log2 2-fold-change in abundance with p < 0.01) are highlighted in red and blue, respectively. The absolute numbers and percentages of significantly modulated ligands are specified in the corresponding quadrants. (C, D) Volcano plots comparing HLA ligand abundances on lenalidomide-treated vs. untreated cells at t24h and t48h, respectively. (E, F) Control volcano plots comparing HLA ligand abundances on untreated cells at t24h and t48h to baseline levels before treatment. (G) Distribution of HLA restrictions among peptides identified on lenalidomide-treated (n = 4,221 peptides) vs. untreated (vehicle controls and pre treatment) primary CLL cells (n = 3,577 peptides). Abbreviations: rep, replicate; vs., versus; FC, fold-change.
thus results in more robust quantitation of peptides across conditions even in runs with lower numbers of FDR-filtered peptide identifications. The identified HLA ligand sequences – based on high-quality peptide spectrum matches and 5% FDR – were queried among all runs without applying any filtering for spectral quality criteria (XCorr, FDR) to extract areas for IDs not passing these thresholds. Using this label-free quantitation (LFQ) strategy, we semi-quantitatively assessed HLA class I ligand presentation during in vitro lenalidomide treatment. We observed no relevant plasticity of the HLA class I ligandome of UPN1 after treatment with lenalidomide (0.03% upmodulation, 0.00% downmodulation, mean of three biological replicates) at t24h compared with untreated controls (Fig. 3C, single biological replicate analysis Fig. S3A). At t48h, similar proportions of modulation were observed (0.00% upmodulation, 0.03% downmodulation, Fig. 3D, single biological replicate analysis Fig. S3B). The only HLA ligands that showed significant alteration in their abundance under lenalidomide treatment are the IKZF1-derived peptide APHARNGLSL419–428 (upmodulation at t24h) and the IL1B-derived peptide SVDPKNYPK200–208 (downmodulation at t48h).

Strikingly, plotting HLA ligand presentation of untreated UPN1 controls at t24h and t48h compared with levels of cells before lenalidomide treatment yielded even higher proportions of modulated HLA class I ligands, with 3.04% (2.96% upmodulation, 0.08% downmodulation) and 6.30% (4.15% upmodulation, 2.15% downmodulation) of HLA ligands significantly altered in their abundance at t24h and t48h, respectively (Figs. 3E and F, single biological replicate analysis Figs. S3C and D).

Gene ontology enrichment analysis (PANTHER version 11.1) comparing the source proteins of all identified HLA ligands on UPN1 with the up and downmodulated source proteins identified in the volcano plot analysis using single biological replicates showed no significant protein enrichment of distinct biological processes. Lenalidomide treatment of UPN2 and UPN3 resulted in similarly low HLA ligandome plasticity (Figs. S4C and F), which confirms that lenalidomide has no relevant influence on the HLA ligands identified on lenalidomide-treated and untreated samples were detected (Fig. 3G).

**In vitro lenalidomide mediates no substantial quantitative or qualitative influence on the HLA class II ligandome of primary CLL cells**

Because of the important role of CD4+ T cells in anticancer immune responses, optimal target selection for T-cell-based immunotherapy may benefit from the inclusion of HLA class II epitopes. For the selection of such epitopes, it is thus also necessary to map the effects of lenalidomide on the HLA class II ligandome. We identified a total of 6,767 unique HLA class II ligands representing 1,642 source proteins on primary CLL cells (n = 3, Fig. 4A, Supplemental Data 4–6). Within this data set, we were able to detect 92 unique CLL-associated HLA class II epitopes described in a previous study (Fig. 4A). A first basic overlap analysis suggested considerable qualitative differences in the composition of HLA class II ligandomes on untreated versus lenalidomide-treated primary CLL cells. However, none of the 417/3,631 (11.5%) and 524/3,755 (14.0%) HLA class II ligands of UPN1 and 3 that showed exclusive presentation on lenalidomide-treated cells (Figs. 4B and S6A) was found to reach the significance thresholds calculated based on permutation analysis (Figs. 4C and S8B). Implementing LFQ using matching between runs, we further semi-quantitatively assessed HLA class II ligand presentation during in vitro lenalidomide treatment. We observed no relevant plasticity of the HLA class II ligandome of UPN1 after treatment with lenalidomide with 0.11% and 0.06% of UPN1 ligands (mean of three biological replicates) showing significant modulation at t24h and t48h, compared with untreated controls, respectively (Figs. 4D and E, single biological replicate analysis Figs. S5A and B). HLA ligand presentation of untreated UPN1 cells compared with the levels of cells before lenalidomide treatment yielded even higher proportions of modulated HLA class II ligands, with 3.05% and 6.54% of HLA ligands significantly altered in their abundance at t24h and t48h, respectively (Figs. 4F and G, single biological replicate analysis Figs. S5C and D). In vitro lenalidomide treatment of UPN3 resulted in similar HLA ligandome plasticity (Figs. S6B and S7), which confirms that lenalidomide has no relevant influence on the relative abundances of HLA class II-presented peptides of primary CLL cells.

**CLL-associated HLA ligands show robust presentation under lenalidomide treatment**

As stable presentation of CLL-associated antigens is indispensable for the rational combination of lenalidomide with T-cell-based immunotherapy approaches, we next longitudinally analyzed the presentation kinetics of established CLL-associated HLA class I and class II ligands upon lenalidomide treatment. For UPN1, 18/27 HLA class I and 29/37 HLA class II ligands could be longitudinally analyzed, as for these peptides MS2 identifications and quantifiable precursor extracted ion chromatograms were available for at least one replicate at each time point. CLL-associated HLA class I ligands showed only slight modulation due to lenalidomide treatment with median log2 fold-change (treated/untreated, mean of three replicates) of 0.28 (range –0.24 to 1.11) and 0.41 (range 0.21 to 1.00) at t24h and t48h, respectively (Fig. 5A, Table S3). Tracking of these specific peptides in single replicate volcano plot analyses confirmed for 17/18 (94.4%) of these CLL-associated HLA ligands that the observed modulation did not reach statistical significance (Figs. S3A and B). Similar results were obtained for CLL-associated HLA class II ligands with median log2 fold-change of −0.01 (range −0.81 to 1.23) and −0.22 (range −1.05 to 0.41) at t24h and t48h, respectively (Fig. 5B, Table S4). 26/29 (89.7%) of these HLA class II ligands showed non-significant modulation in the single replicate volcano plot analysis (Figs. S5A and B), confirming the robust presentation of the CLL-associated antigens under lenalidomide exposure.

**IKZF1- and IKZF3-derived HLA ligands are selectively and significantly upmodulated under lenalidomide treatment of primary CLL cells**

As the IKZF1-derived HLA class I ligand APHARNGLSL419–428 showed significant upmodulation under lenalidomide...
Figure 4. Quantitative and qualitative influence of *in vitro* lenalidomide treatment on the HLA class II peptidome of primary CLL cells. (A) Overview of PBMC count, unique HLA class II ligand IDs, corresponding source proteins and the number of unique CLL-associated antigens identified by mass spectrometry of analyzed primary CLL samples (n = 3). (B) Overlap analysis of HLA class II ligands identified on lenalidomide-treated vs. untreated (vehicle controls and pre treatment) cells (UPN1). (C) Frequency-based analysis of peptide presentation on treated vs. untreated (vehicle controls and pre treatment) UPN1 cells. HLA class II ligands are indicated on the x-axis, the frequency of positive ligandomes on the y-axis. (D–G) Volcano plots of modulation in the relative abundances of HLA class II ligands on UPN1 cells comparing the conditions indicated. Each dot represents a specific HLA ligand. Log2 fold-changes of peptide abundance are indicated on the x-axis, the corresponding significance levels after multi-testing correction (−log10 p-value) on the y-axis. HLA ligands showing significant up or downmodulation (≥ log2 2-fold-change in abundance with p < 0.01) are highlighted in red and blue, respectively. The absolute numbers and percentages of significantly modulated ligands are specified in the corresponding quadrants. (D, E) Volcano plots comparing HLA ligand abundances on lenalidomide- vs. untreated cells at t24h and t48h, respectively. (F, G) Control volcano plots comparing HLA ligand abundances on untreated cells at t24h and t48h to baseline levels before treatment. Abbreviations: UPN, uniform patient number; rep, replicate; vs., versus; FC, fold-change.
treatment (UPN1, Fig. 3C), we aimed to analyze whether the direct inhibition of CLL cell proliferation via cereblon caused by lenalidomide is reflected in the HLA ligandome of treated primary CLL cells. We screened the HLA ligandome of UPN1 for the presence of IKZF1- and IKZF3-derived ligands, as these two transcription factors undergo increased proteasomal degradation under lenalidomide treatment.34,43 We identified two length variants of an IKZF1-derived HLA class I ligand (APHARNGLSL419–428, B*07:02; APHARNGL419-426, B*07:02) and one IKZF3-derived HLA class I ligand (AEMGSERAL246–254, B*44:02). Strikingly, the IKZF3-derived ligand was detected exclusively on CLL cells after lenalidomide treatment and the IKZF1-derived ligands showed substantial upmodulation under lenalidomide treatment with median log2 fold-changes (treated/untreated, mean of three replicates) of 1.25 and 1.72 at t24h and t48h, respectively (Fig. 5C). In the single replicate volcano plot analysis comparing the HLA ligandomes of lenalidomide-treated cells with cells before treatment, the IKZF1-derived ligand APHARNGLSL reached significance thresholds (log2 fold-change ≥ 2, p ≤ 0.01 after multi-testing correction) for upmodulation in 2/3 biological replicates at both, t24h and t48h (Figs. 5D and E).

### Discussion

The positive effects of the immunomodulatory drug lenalidomide on antibody-dependent NK cell-mediated cytotoxicity,44 antigen presentation by dendritic cells22 as well as T-cell function and activation23,24 suggest that lenalidomide is a promising compound for combination with T-cell-based immunotherapeutic approaches.45 As precise and effective antigen-specific cancer immunotherapy requires the exact knowledge of presentation patterns and kinetics of tumor-associated HLA-presented epitopes that can act as rejection antigens, a potential impact of lenalidomide on antigen presentation of target cells would be of paramount interest. To our knowledge, this is the first study that evaluates the influence of lenalidomide on the HLA-presented immunopeptidome of primary cancer cells. Our previous studies indicated that a mass spectrometry-based approach is highly efficient in longitudinally mapping the effect of anticancer drugs on the HLA ligandome32 as well as for identification of physiologic targets of anticancer T-cell responses in patients with hematological malignancies.27,46,47 In CLL, this strategy enabled us to establish a panel of highly specific, immunogenic and...
pathophysiologically relevant CLL-associated antigens, which may be implemented for antigen-specific immunotherapy. However, profound immune defects in CLL patients might constitute a major limitation for such T-cell-based approaches. Combination of specific T-cell-based approaches with the immunomodulatory agent lenalidomide may represent a suitable option to overcome the immunosuppressive state in CLL. The present study was designed to analyze the impact of lenalidomide on the HLA-presented antigenic landscape of primary CLL cells, thereby allowing the informed selection of robustly presented targets for antigen-specific immunotherapy. We used the dose of 0.5 μM of lenalidomide for in vitro treatment of primary CLL cells, which was previously shown to be adequate to induce the reported positive effects on the microenvironment of CLL, especially on T cells. To take into account the kinetics of HLA peptide processing and presentation, we implemented two time points of longitudinal HLA ligand analysis at 24h and 48h. We found that lenalidomide does not cause a significant alteration of HLA class I and II surface expression on CLL and autologous B cells in contrast to several other anticancer drugs. Furthermore, no relevant alterations in the HLA allotype distribution or in the presentation of previously defined CLL-associated HLA ligands were detected, which enables a straightforward target selection in CLL patients irrespective of lenalidomide treatment. Recent data demonstrated the induction of novel, cryptic, treatment-associated HLA ligands for anticancer drugs like decitabine and carfilzomib. In this study, we demonstrate that lenalidomide does not significantly induce cryptic, treatment-associated antigens. However, we detected a significant upmodulation of IKZF1-derived HLA-presented peptides under lenalidomide exposure, which might be due to the direct effect of lenalidomide on CLL cells via cereblon-driven proteasomal degradation of this transcription factor. Further studies will be needed to evaluate the impact of IKZF1- and IKZF3-induced peptides under lenalidomide treatment, especially concerning their eligibility as novel T-cell epitopes.

Together, our study shows that in vitro lenalidomide has no relevant influence on the HLA-presented immunopeptidome of primary CLL cells and therefore adds novel important aspects toward characterizing lenalidomide as a suitable agent for the combination with T-cell-based immunotherapy. Based on these results, we implemented a phase II peptide vaccination study combining our CLL-associated HLA ligands with lenalidomide treatment following first-line therapy of CLL patients (NCT02802943).

Material and methods

Patients and blood samples

Peripheral blood mononuclear cells (PBMCs) from CLL patients (≥88% CLL cells) before therapy were isolated by density gradient centrifugation (Biocoll, L 6113). Informed written consent was obtained in accordance with the Declaration of Helsinki protocol. The study was performed according to the guidelines of the local ethics committee (373/2011BO2). Patient characteristics are provided in Table S1.

In vitro lenalidomide treatment of primary CLL samples

Primary CLL samples were cultured in RPMI1640 medium (life technologies, 10270-106) supplemented with 10% fetal bovine serum (life technologies, K 0293) and 1% sodium pyruvate (Biochrom, L 0473) and incubated with lenalidomide (0.5 μM, Selleckchem, S1029) for 24 or 48h (t24h and t48h). Controls were incubated with 0.005% DMSO as vehicle control for 24 or 48h. Cell viability analysis was performed using trypan blue exclusion staining. Experiments were conducted in three biological replicates where indicated. Therefore, isolated PBMCs of the same blood sample were split into three portions for each condition and treated in parallel. Please note that one HLA class I data set (UPN1 untreated #3 t24h) did not pass the quality control threshold of 500 ligands for being included in LFQ analysis and had to be replaced by UPN1 untreated #2 t24h. All analyses based on LFQ data therefore implement UPN1 untreated #2 compared with lenalidomide #3 as the data set #3 for lenalidomide-induced modulation at t24h. For the HLA class I data set of UPN2, the mass spectrometry analysis could only be performed at t24h because of technical problems during the measurement of the lenalidomide t48h data set. Due to a low number of HLA class II ligands identified for UPN2 (mean: 149 HLA class II ligands per condition) this data set was excluded from the analyses. Sample characteristics including cell count, cell viability and HLA class I and II ligand IDs are provided in Table S2.

Quantification of HLA surface expression

HLA surface expression on CD19+CD5+ CLL cells and CD19+CD5− autologous B cells of CLL patients was analyzed using the QIIFIKIT bead-based quantitative flow cytometric assay (Dako, K0078) according to manufacturer’s instructions as described before. In brief, samples were stained with the pan-HLA class I-specific monoclonal antibody (mAb) W6/32 (produced in-house), the HLA-DR-specific mAb L243 (produced in-house) or IgG isotype control (BioLegend, 400202), respectively. Surface marker staining was performed with directly labeled APC anti-human CD19 (BioLegend, 202212), PE anti-human CD5 (BioLegend, 300608) and APC-H7 anti-human CD3 (BD, 641406) antibodies. Aqua fluorescent reactive dye (life technologies, L34957) was used as viability marker. Flow cytometric analysis was performed on a FACSCanto II Analyzer (BD).

Isolation of HLA ligands from primary CLL samples

HLA class I and class II molecules were isolated using standard immunoaffinity purification as described before, using the pan-HLA class I-specific mAb W6/32, the pan-HLA class II-specific mAb Tü 39, and the HLA-DR-specific mAb L243 (all produced in-house) to extract HLA ligands. All samples of each patient were adjusted to identical cell counts before HLA ligand isolation.
Analysis of HLA ligands by LC-MS/MS

HLA ligand extracts were analyzed in five technical replicates as described previously. In brief, peptide samples were separated by nanoflow high-performance liquid chromatography (RSLCnano, Thermo Fisher Scientific) using a 50 μm × 25 cm PepMap rapid separation liquid chromatography column (Thermo Fisher Scientific) and a gradient ranging from 2.4% to 32.0% acetonitrile over the course of 90 min. Eluting peptides were analyzed in an online-coupled LTQ Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) using a top speed collision-induced dissociation fragmentation method for samples of UPN1 and UPN3. Samples of UPN2 were analyzed in an online-coupled LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) using a top five collision-induced dissociation fragmentation method.

Database search and HLA annotation

Data processing was performed as described previously. In brief, for UPN1 and UPN3 (orbitrap fragment spectra), the SEQUEST HT search engine (University of Washington) and for UPN2 (ion trap fragment spectra) the Mascot search engine (Mascot 2.2.04; Matrix Science) were used to search the human proteome as described previously. Enzymatic digestion of peptides was performed using trypsin with a 1:100 (w/w) enzyme to peptide ratio. Peptide sequences were limited to 12 amino acids for HLA class I and 25 amino acids for HLA class II. Protein inference was performed with a minimum of five peptides. MS/MS spectra were searched against the UniProt/Swiss-Prot database (20,279 reviewed protein sequences, September 27th 2013) with the criteria that precursor mass tolerance was set to 5 ppm, and fragment mass tolerance to 0.5 Da for ion trap spectra analyzed by Mascot and 0.02 Da for orbitrap spectra analyzed by SEQUEST HT, respectively. Oxidized methionine was allowed as a dynamic modification. The false discovery rate (FDR) was estimated using the Percolator algorithm and limited to 5% for HLA class I and 1% for HLA class II. Protein inference was disabled, allowing for multiple protein annotations of peptides. HLA class I and class II annotation was performed using NetMHCpan 3.0 and NetMHCIIpan 3.1. Annotating peptides with IC50 scores or percentile rank below 500 nM or 2%, respectively. In cases of multiple possible annotations, the HLA allotype yielding the lowest rank/score was selected.

Label-free quantitation of HLA ligand presentation

For LFQ of the relative abundances of HLA ligands over the course of lenalidomide treatment, the total cell numbers of all samples per patient were normalized by adjusting the implemented volume of cell lysate before HLA ligand isolation. LC-MS/MS analysis was performed in five technical replicates for each sample. 500 HLA ligands per sample in five merged technical replicates were set as threshold for the inclusion of the sample in LFQ analysis. Relative quantification of HLA ligands was performed based on the area of the corresponding precursor extracted ion chromatograms using ProteomeDiscoverer 1.4.1.14 (Thermo Fisher Scientific). Peptide identifications and their measured intensities are provided in supplemental data 1–6. Reproducibility of quantitation across biological replicates in the LFQ strategy using matching between runs is shown in a correlation analysis of the MS-measured intensities of identified HLA ligands (Fig. S2). To cope with the common problem of missing values in DDA MS and label-free quantitative proteomics, we used a LFQ strategy using matching between runs to reduce missing values in quantitation by lowering FDR cutoffs. High-quality peptide spectrum matches filtered for 5% FDR and subsequently screened for predicted HLA binding (netMHCpan 3.0 rank < 2% or affinity < 500 nM) were used to generate seed lists for semi-quantitative volcano plot analysis. The sequences from these seed lists were then queued across all runs without applying any filtering for spectral quality criteria (XCorr, FDR) to extract areas for IDs not passing these thresholds. For Volcano plot analysis, “one hit wonders” (peptides only found in one technical replicate) were discarded and the sample-specific limit of detection (LOD) was calculated as the median of the five lowest areas and inserted for missing areas to allow for fold-change calculation of HLA ligands detected in only one of both conditions. Technical replicates as well as experiments/conditions were normalized based on the summed intensities of all identified precursors in each MS run. Subsequently, the ratios of the mean areas of the individual peptides in the five LFQ-MS runs of each sample were calculated and unpaired, heteroskedastic two-tailed t-tests implementing Benjamini–Hochberg correction were performed using an in-house R script (v3.2.3).

Software and statistical analysis

Flow cytometric data analysis was performed using FlowJo 10.0.7 (Treestar). An in-house Python script was used for permutation analysis for the calculation of FDRs of treatment-associated peptides at different presentation frequencies, as described previously. Briefly, the numbers of original treatment-associated peptides identified based on the analysis of the treated and untreated samples were compared with random simulated treatment-associated peptides. Simulated treated and untreated samples were generated based on random weighted sampling from the entirety of peptide identifications in both original conditions. These randomized virtual ligandomes were used to define virtual treatment-associated peptides based on simulated cohorts of treated versus untreated samples. The process of peptide randomization, cohort assembly and identification of treatment-associated peptides was repeated 1,000 times and the mean values of resultant decoy identifications as well as the corresponding FDRs for any chosen frequency of treatment-exclusive peptide presentation were calculated. Of note, due to the limited number of biological replicates in UPN2 and UPN3 permutation analysis could only be performed for UPN1. In-house R scripts were used for volcano plots and longitudinal analysis of relative HLA ligand abundances. The longitudinal analysis of relative HLA ligand abundances utilizes the mean of three biological replicates. Overlap analysis were performed using BioVenn. GraphPad Prism 6.0 (GraphPad Software) was used for statistical analysis. Comparative analysis of HLA surface expression was based on unpaired t-tests.
Abbreviations

CLL chronic lymphocytic leukemia  
DDA data-dependent acquisition  
DMSO dimethyl sulfoxide  
FC fold-change  
FDR false discovery rate  
HLA human leukocyte antigen  
IKZF1 ikaros family zinc finger protein 1  
IKZF3 ikaros family zinc finger protein 3  
IL1B interleukin-1 β  
LC-MS/MS liquid chromatography-coupled tandem mass spectrometry  
LFQ label-free quantitation  
LTQ linear trap quadrupole  
mAb monoclonal antibody  
MS mass spectrometry  
ns not significant  
PBMCs peripheral blood mononuclear cells  
rep replicate  
SD standard deviation  
TRQ threshold for relative quantitation  
UPN uniform patient number

Disclosure of potential conflicts of interest

Daniel J. Kowalewski and Heiko Schuster are employees of Immatics Biotechnologies GmbH. Hans-Georg Ramenese is shareholder of Immatics Biotechnologies GmbH and Curevac AG. The other authors declare no competing financial interests.

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