Identification of an immunogenic DKK1 long peptide for immunotherapy of human multiple myeloma

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ABSTRACT

Dickkopf-1 (DKK1), broadly expressed by tumor cells from human multiple myeloma (MM) and other cancers but absent from most normal tissues, may be an ideal target for immunotherapy. Our previous studies have shown that DKK1 (peptide)-specific cytotoxic T lymphocytes can effectively lyse primary MM cells in vitro. To develop DKK1-based vaccines that can be easily and inexpensively made and used by all patients, we identified a DKK1 long peptide (LP), DKK1<sub>3-76</sub>-LP, that contains 74 amino acids and epitopes that can potentially bind to all major MHC class I and II molecules. Using HLA-A*0201- and HLA-DR*4-transgenic mouse models, we found that DKK1-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses, detected by DKK1 short peptide (P<sub>20</sub> and P<sub>66v</sub>)-HLA-A*0201 tetramer staining and cytotoxic assay for CD8<sup>+</sup> T cells or by carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution and IFN-γ secretion for CD4<sup>+</sup> T cells, respectively, can be induced in vivo by immunizing mice with the DKK1<sub>3-76</sub>-LP. In addition, DKK1<sub>3-76</sub>-LP also induced anti-DKK1 humoral immunity in the transgenic mice and the DKK1 antibodies were functional. Finally, DKK1<sub>3-76</sub>-LP stimulated human blood T cells ex vivo to generate DKK1-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses from 8 out of 10 MM patients with different MHC backgrounds. The generated DKK1-specific CD8<sup>+</sup> cells efficiently lysed autologous MM cells from these patients. Thus, these results confirm the immunogenicity of the DKK1<sub>3-76</sub>-LP in eliciting DKK1-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses in vitro and in vivo, and suggest that the DKK1<sub>3-76</sub>-LP can be used for immunotherapy of MM and other cancers.

Introduction

Dickkopf-1 (DKK1) is highly expressed in tumor cells of multiple myeloma (MM) and other cancer types, but is absent from normal tissues and organs, with the exception of the placenta and prostate. Our previous studies have shown that DKK1 (peptide)-specific cytotoxic T lymphocytes (CTL) can effectively lyse primary myeloma cells in vitro, confirming that DKK1 may be a good tumor-associated antigen. We explored the efficacy of a murine DKK1 DNA vaccine in the murine MOPC-21 myeloma model and showed that active vaccination using the DKK1 vaccine was not only able to protect mice from developing myeloma, but was also therapeutic against established myeloma. Mechanistic studies revealed that the DKK1 vaccine elicited strong DKK1- and tumor-specific CD4<sup>+</sup> and CD8<sup>+</sup> immune responses. Thus, our studies provide a strong rationale for targeting DKK1 for immunotherapy in myeloma patients.

There has been substantial progress in the clinical use of long peptide (LP) therapeutic vaccination in recent years. Melief et al. reported an LP vaccine encompassing
a CTL epitope and possessing immunotherapeutic potential. Disis et al. reported that vaccination with a CTL-epitope LP derived from human epidermal growth factor receptor 2 (EGFR2, better known as HER2) generated robust and persistent tumor-specific T-cell immunity in patients with metastatic breast cancer. Recent clinical studies using a telomerase-derived LP encompassing CTL-epitopes (CV1001) showed an increase in survival of cancer patients when given in combination with radio- and chemotherapy. The success of LP therapeutic vaccines can be attributed to the fact that it could induce close collaboration between cells of the innate immune system, in particular antigen-presenting dendritic cells (DC) and cells of the adaptive immune system, especially CD4+ T-helper (Th) cells and CD8+ CTL. LP must be taken up and processed by antigen-presenting cells (APC) before they are presented. Professional APC, such as DC, can manage pools of LP and are capable of properly exciting multiple HLA class I and II peptide epitopes for presentation at the cell surface.

Therefore, injection of LP will ensure the induction of both CD4+ and CD8+ T cells to available epitopes, each of which can contribute to the anti-tumor response. CD8+ CTL exert key cytotoxicity to tumor cells. CD4+ T cells are necessary elements of cellular immunity for priming tumor-specific CTL and influencing the differentiation and expansion of tumor antigen-specific CTL. Thus, an ideal peptide vaccine for cancer immunotherapy may be optimally composed of a single LP spanning epitopes for both Th cells and CTL.

In this study, we identified a human DKK1-derived LP (DDK1L37-76-LP) and explored its potential as a vaccine to induce human DKK1-specific CD4+ Th and CD8+ CTL responses. We found that DDK1L37-76-LP successfully induced Th1-cell responses in individuals expressing several common HLA allelic variants, including HLA-A, HLA-B, HLA-C, HLA-DR alleles, and that an efficient cross-presentation of the DDK1L37-76-LP also induced DKK1-specific CTL response.

### Methods

#### Patients and samples

Peripheral blood samples from patients with MM and healthy donors were used. This study was approved by the Institutional Review Board of the Cleveland Clinic, and informed consent was obtained in accordance with the Declaration of Helsinki.

#### Selection of HLA class I and II binding peptides

To predict possible promiscuous HLA class I and II-binding peptides on human DKK1, the amino acid sequence of the human DKK1 protein was analyzed by Immune Epitope Database (IEDB) recommended methods. The program identified a 74 amino acid LP, DDK1L37-76, that contains multiple peptide motifs (Figure 1) with high affinity for common and major MHC class I and class II molecules, representing 95% of humans.

All peptides, including long and short MHC class I and class II binding peptides, were synthesized by Biosynthesis (Lewisville, TX, USA). The purity of synthetic peptides, confirmed by reversed-phase high-performance liquid chromatography and mass spectrometry, was over 98%. Synthetic peptides were dissolved in dimethyl sulfoxide (DMSO; Sigma, St Louis, MO, USA), and stored at -20°C until use.

#### Generation of dendritic cells

Monocyte-derived mature DC were generated from human peripheral blood mononuclear cells (PBM C). The quality of DC was judged based on their expression of CD11c, CD40, CD80, CD86, and MHC class II molecules. Detailed information is provided in the Online Supplementary Appendix.

### Determination of in vivo immunogenicity of DKK1 peptides

HLA-A*0201-transgenic (Tg[HLA-A2.1]) and HLA-DR4-transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained at the animal facility and studies were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

For immunization, peptides were diluted in phosphate buffered saline at room temperature, mixed, and emulsified with an equal volume of incomplete Freund’s adjuvant. Groups of three mice were immunized subcutaneously at the tail base with 100 L of emulsion containing 100 g of peptides. All of the mice were immunized at least three times. Two weeks after the immunization, mice were killed and splenocytes were isolated for in vitro studies. The same experiments were repeated three times.

### Generation of DKK1-specific CD4+ and CD8+ T-cell responses

DDK1-specific T cells were generated from PBMC of HLA-A*0201 and HLA-DR4 blood donors and patients with MM by repeated stimulations of autologous T cells with DKK1 peptide-loaded mature DC. Further details are available in the Online Supplementary Appendix.

#### Cytotoxicity assay

The standard 7-AAD/CFSE Cell-Mediated Cytotoxicity Assay Kit was used to measure the cytolytic activity of T cells on target cells. Further details are available in the Online Supplementary Appendix.

### Statistical analyses

Statistical analysis was performed with Student t-test. P<0.05 was considered statistically significant. Results are presented as mean ± standard deviation unless otherwise indicated.

### Results

#### Identification of long peptide containing multiple T-cell epitopes on Dickkopf-1 protein

To identify LP comprising the most potential MHC class I and II binding epitopes on human DKK1 protein, we examined the amino acid sequence of DKK1 using the following websites: http://www.bimas. cii. nih. gov/molbio/hla bind/ and http://www.imtech. res. in/raghava/propred/; www.immunoeptide. org to predict the epitopes. We focused on regions with multiple MHC class I and class II epitope binding prospects. As a result, we identified an LP, DDK1L37-76, that contains 74 amino acids and multiple epitopes that can potentially bind with all major MHC class I (e.g., HLA-A, B, or C) and class II molecules (e.g., HLA-
DR1, -DR4, or -DR7) (Table 1 and Figure 1). DKK1\textsubscript{3-76}-LP contains our previously identified HLA-A*0201-restricted T-cell epitopes DKK1-P20 and DKK1-P66v.\textsuperscript{19}

**In vivo immunogenicity of the Dickkopf-1\textsubscript{3-76}-long peptide in activating Dickkopf-1-specific CD8\textsuperscript{T} cytotoxic T lymphocytes**

To assess the immunity of the DKK1\textsubscript{3-76}-LP in inducing CD8\textsuperscript{T} CTL response *in vivo*, we used HLA-A*0201-transgenic mice and immunized them four times with either DKK1\textsubscript{3-76}-LP or DKK1-P20 short peptide. The capacity of DKK1\textsubscript{3-76}-LP to prime DKK1-specific CTL response was examined using HLA-A*0201-P20- (Figure 2A) or HLA-A*0201-P66v tetramer staining (Figure 2B). The results clearly showed that mice immunized with DKK1-P20 short peptide (Figure 2A and B, top panels) had increased percentages of DKK1-P20 (P<0.01, compared with control), but not DKK1-P66v, tetramer\textsuperscript{CD8\textsuperscript{T}} T cells in the spleen after each round of immunization, whereas mice immunized with DKK1\textsubscript{3-76}-LP (Figure 2A and B, bottom panels) generated CD8\textsuperscript{T} T-cell response against both DKK1-P20 and DKK1-P66v (P<0.01, compared with control). Moreover, CD8\textsuperscript{T} T cells isolated from mice immunized with DKK1\textsubscript{3-76}-LP were not only able to kill syngeneic DC pulsed with DKK1\textsubscript{3-76}-LP, but also DC pulsed with DKK1-P20 short peptide (P<0.01, compared with controls) (Figure 2C). Further more, these CD8\textsuperscript{T} T cells also killed HLA-A*0201+ U266, but not HLA-A*0201 ARP-1 myeloma cells or K562 cells (to exclude natural killer [NK]-cell activity) (P<0.01, compared with controls) (Figure 2D). Hence, these results demonstrate that DC, after uptake of DKK1\textsubscript{3-76}-LP, efficiently cross-present T-cell epitopes on the DKK1\textsubscript{3-76}-LP and activate CTL specific for various T-cell epitopes, including DKK1-P20- and DKK1-P66v, *in vivo*.

**In vivo immunogenicity of the Dickkopf-1\textsubscript{3-76}-long peptide in activating Dickkopf-1-specific CD4\textsuperscript{T} helper cells and antibody production**

Next, we assessed whether DKK1\textsubscript{3-76}-LP could also elicit DKK1-specific CD4\textsuperscript{T} Th cell response. HLA-DR\textsuperscript{4}-transgenic mice were available commercially and immunized

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**Table 1. Potential Dickkopf-1 peptides for different MHC molecules.**

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**Table 1. Potential Dickkopf-1 peptides for different MHC molecules.**

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\textsuperscript{a}http://www.bimas.cit.nih.gov/molbio/hla_bind  \textsuperscript{b}http://www.imtech.res.in/raghava/propred/  \textsuperscript{c}http://www.immune epitope.org

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four times with DKK1$_{3-76}$-LP or a HLA-DR*4-restricted and -binding DKK1-P$_{30}$ short peptide (Table 1). CD4$^+$ T-cell response was detected by CFSE dilution and IFN-γ secretion. The results clearly showed that mice immunized with either DKK1$_{3-76}$-LP or DKK1 F$_{30}$ short peptide had significantly higher percentages of proliferating CD4$^+$ T cells in the spleen after ex vivo re-stimulation with DC pulsed, but not unpulsed, with DKK1$_{3-76}$-LP or DKK1-F$_{30}$ short peptide ($P < 0.01$, compared with non-immunized mice) (Figure 3A). Moreover, splenocytes isolated from DKK1$_{3-76}$-LP - or DKK1 F$_{30}$-immunized mice contained significantly more CD4$^+$ IFN-γ-expressing cells after ex vivo re-stimulation with DKK1$_{3-76}$-LP - or DKK1 F$_{30}$-, respectively, pulsed, but not unpulsed, DC than non-immunized mice ($P < 0.01$, compared with those from non-immunized mice) (Figure 3B). Interestingly, the percentages of Foxp3$^+$CD4$^+$ T cells were similarly low in mice with or without peptide immunization (Figure 3B), indicating that DKK1$_{3-76}$-LP vaccination induced DKK1-specific CD4$^+$ T-cell responses without promoting regulatory T-cell (Treg) formation in vivo. Taken together, these results demonstrate that the DKK1$_{3-76}$-LP is immunogenic in vivo to induce DKK1-specific CD4$^+$ and CD8$^+$ T-cell responses.

We also investigated whether the DKK1$_{3-76}$-LP could induce a DKK1-specific humoral immune response and examined whether there were DKK1-specific antibodies in the sera of DKK1$_{3-76}$-LP-immunized HLA-A*0201- or HLA-DR*4-transgenic mice. ELISA results showed that high titers of DKK1-specific IgG antibodies were detected in HLA-A*0201-transgenic mice immunized with DKK1$_{3-76}$-LP, but not with DKK1 F$_{30}$ short peptide, and the titers of the antibodies increased after each cycle of immunization ($P < 0.01$, compared with DMSO control) (Figure 4A). Similarly, DKK1-specific antibodies were also detected in the sera of HLA-DR*4-transgenic mice immunized with DKK1$_{3-76}$-LP but not the DKK1-F$_{30}$ short peptide ($P < 0.01$, compared with DMSO control) (Figure 4B).

Next, we determined whether the detected DKK1-specific antibodies in DKK1$_{3-76}$-LP-immunized mice were biologically functional. As DKK1 was shown to inhibit human osteoblast differentiation, we used an in vitro osteoblast culture system to determine the function of the immunized sera on osteoblast formation in the presence of recombinant human DKK1. The results showed that commercially obtained DKK1-specific antibodies and sera from DKK1$_{3-76}$-LP-immunized transgenic mice abolished DKK1-induced inhibition of human osteoblast differentiation (Figure 4C). These results indicate that the DKK1$_{3-76}$-LP encompasses naturally processed B-cell epitopes, and antibodies generated by DKK1$_{3-76}$-LP immunization are able to neutralize DKK1.

**Immunogenicity of the Dickkopf-1$_{3-76}$-long peptide in priming human Dickkopf-1-specific T cells ex vivo**

Next, we investigated whether DKK1$_{3-76}$-LP was able to induce human DKK1-specific T-cell responses ex vivo. Freshly prepared PBMC from healthy donors or myeloma patients were stimulated with DKK1$_{3-76}$-LP every week. The presence and frequency of DKK1-specific T cells were detected by flow cytometry. Figure 5A shows an increased frequency of DKK1-specific, IFN-γ-secreting CD4$^+$ and CD8$^+$ cells in cultured T cells during in vitro (re)-stimulation with the LP. Figure 5B shows the percentages of HLA-A*0201-DKK1-P$_{20}$ tetramer$^+$ CD8$^+$ T cells in cultures after repeated stimulations with DKK1$_{3-76}$-LP. By using monoclonal antibodies (mAb) specific to HLA-DR or -DQ or HLA-ABC added to T-cell cultures before assay, we showed that MHC class II-restricted CD4$^+$ T cells were...
the main IFN-γ-secreting cells, because anti-HLA-DR mAb significantly reduced the percentage of IFN-γ-secreting CD4+ cells (Figure 5C). Moreover, HLA-A2 antibody significantly reduced the percentages of IFN-γ-secreting CD8+ T cells in cultures after repeated stimulations with DKK113-76-LP. The results showed that DKK113-76-LP-specific tetramer staining showing the frequency of DKK113-76-LP-specific CD8+ T cells in the spleen of an HLA-A*0201-transgenic mouse. Representative results from one of three mice are shown. (B) DKK113-76-LP-specific tetramer staining showing the frequency of DKK113-76-LP-specific CD8+ T cells in the spleen of an HLA-A*0201-transgenic mouse. Representative results from one of three mice are shown. (C) Cytolytic activity of CD8+ T cells isolated from mice immunized with DKK113-76-LP against unpulsed dendritic cells (DC) or DC pulsed with DKK113-76-LP or P20 short peptide. Representative results of three experiments are shown. (D) Cytolytic activity of CD8+ T cells isolated from mice immunized with DKK113-76-LP against U266 and ARP-1 human myeloma cell lines, and K562. Representative results of three experiments are shown. *P<0.05; **P<0.01.

Discussion

In this study, we identified a 74aa DKK113-76-LP that contains multiple epitopes for CD4+ and CD8+ T cells and explored the potential of using this LP for immunotherapy of human MM. We showed that DKK113-76-LP-specific CTL, detected by DKK1 short peptide (P20 and P66v)-HLA-A*0201 tetramer staining, and DKK113-76-LP-specific CD8+ T cells were detected by intracellular IFN-γ staining. Figure 6A is a representative flow cytometry analysis showing the frequencies of IFN-γ-secreting CD8+ and CD4+ T cells after re-stimulation with DKK113-76-LP from a MM patient (MM1). Low percentages of IFN-γ-secreting CD8+ and CD4+ T cells were observed in T-cell cultures re-stimulated with unpulsed DC or DC pulsed with DKK113-76-LP or P20 short peptide. Figure 6B shows the percentages of IFN-γ-secreting CD4+ and CD8+ T cells from a total of ten patients with MM (with different MHC backgrounds) after a 4-week in vitro stimulation of blood T cells with DKK113-76-LP-pulsed autologous DC. Furthermore, we generated CD8+ T-cell lines from HLA-A*0201+ patients by in vitro repeated stimulations with DKK113-76-LP and these DKK113-76-LP-specific T cells killed autologous patient (MM1) myeloma cells, primary myeloma cells from another HLA-A*0201 patient (MM2), and HLA-A*0201 myeloma cell line U266. No killing was observed on HLA-A*0201 primary myeloma cells (MM3) or myeloma cell line ARP-1, normal B cells, or K562 cells (to exclude NK-cell activity) (Figure 6C).

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Figure 3. Dickkopf-1 (DKK1)-long peptide (LP) efficiently induces DKK1-specific CD4+ T-cell response in vivo. Shown are CD4+ T-cell responses induced in HLA-DR*4-transgenic mice after immunization with DKK1-1-76-LP or HLA-DR-restricted P30 short peptide. (A) Carboxyfluorescein succinimidyl ester (CFSE) dilution assay showing the percentages of proliferating CD4+ T cells from the spleen of mice after ex vivo re-stimulation with dendritic cells (DC) pulsed, but not unpulsed, with DKK1-1-76 LP or DKK1-1-76 short peptide. Representative results from one of three independent experiments are shown. (B) Flow cytometry analysis showing the percentages of IFN-γ expressing or FoxP3+ CD4+ T cells from the spleen of mice after ex vivo re-stimulation with DC pulsed, but not unpulsed, with DKK1-1-76 LP or DKK1-1-76 short peptide. Representative results from one of four experiments are shown.

Figure 4. Dickkopf-1 (DKK1)-long peptide (LP) induces DKK1-specific humoral immune response in vivo. Serum titers of DKK1-specific antibodies in mice after immunization with DKK1-1-76-LP or short peptide are shown. (A) ELISA showing the titers of DKK1-specific IgG antibodies in HLA-A*0201-transgenic mice immunized with DKK1-1-76 LP or P20. (B) ELISA showing the titers of DKK1-specific IgG antibodies in HLA-DR*4-transgenic mice immunized with DKK1-1-76 LP or P30. (C) Effect of DKK1 on differentiation of human osteoblasts in culture with the addition of control mouse IgG, anti-DKK1 antibody (R&D Systems) or serum from DKK1-1-76 LP vaccinated (after the 4th vaccination) HLA-A*0201-transgenic mice. Results of three experiments are shown. DMSO: dimethyl sulfoxide. *P<0.05; **P<0.01.
ing solid evidence that the phenomenon of epitope spreading is critical to the development of effective anti-cancer immunity elicited by peptide vaccination. These results further implicate functional interactions between vaccine-induced CTL and malignant cells that facilitate the induction of large numbers of tumor-specific CTL, the cytolytic effector immune cells that subsequently destroy tumor cells.

Disis et al. reported that vaccination with a herceptin-2 (HER-2/neu)-derived LP encompassing an HLA-A*0201-restricted CTL epitope elicited embedded epitope-specific CD8+ T cells in cancer patients.8 They showed that tumor-specific CTL can be elicited in vivo via cross-presentation of HER-2/neu-derived LP. Such T-cell responses are considered to be crucial for tumor eradication and for generating long-term memory.23 With this premise in mind, we identified an immunogenic DKK1_{3-76}-LP that encompasses both Th epitopes and CTL-epitopes and demonstrated that cross-presentation of DKK1_{3-76}-LP induced priming and expansion of DKK1-specific CTL in vitro and in vivo. Vaccination with DKK1_{3-76}-LP can potentially elicit combined Th and CTL responses. A recent clinical trial showed that targeting Th cells with DC pulsed with both HLA class I and II-restricted epitopes effectively enhanced vaccine-specific immune responses and improved clinical outcome.24 DKK1_{3-76}-LP bolstered the induction of DKK1-P20-specific CTL derived from both healthy donors and MM patients in vitro. Thus, DKK1_{3-76}-LP administered in combination with DKK1-P20 immunotherapy may be able to augment the elicitation of antigen-specific CTL.

Based on the HLA-subtypes capable of antigen presentation from studies using healthy donors, DKK1-P20 and DKK1_{3-76}-LP are predicted to be useful in approximately 80% of the total population. We showed that DKK1_{3-76}-LP induced HLA-DR5–, HLA-DR8–, or HLA-DR15-restricted Th cells in healthy donors and also induced HLA-DR- or HLA-DQ-restricted Th cells in MM patients. However, these MM patients were negative for HLA-DF5–, -DR8–, or -DR15 alleles. We also showed that DKK1_{3-76}-LP induced HLA-DR15- or HLA-DQ-restricted Th cells in healthy donors. These results suggest that DKK1_{3-76}-LP may encompass Th cell epitopes not previously identified in experiments involving cells derived from healthy donors and DKK1_{3-76}-LP may be broadly useful in the majority of MM patients.

Weide et al. reported that the presence of circulating Th cells responding to melanoma antigens Melan-A or NY-ESO-1 has a strong independent prognostic impact on survival among chemotherapy-treated advanced melanoma patients.25 Another study has shown a possible
synergy between the telomerase-specific Th responses with chemotherapy in lung cancer. The introduction of immunotherapy in clinical practice also emphasized the influence of immune responses on cancer prognosis and chemotherapy effectiveness. Poliout et al. and Terpos et al. reported that serum concentration of DKK1 protein were increased in patients with MM and were correlated with severe bone disease. Autologous stem cell transplantation and chemotherapy with bortezomib, melphalan, dexamethasone and intermittent thalidomide significantly reduced serum DKK1 level and led to normalization of bone remodeling in relapsed myeloma. These pieces of evidence support the hypothesis that induction or augmentation of DKK1-specific Th1 cells by vaccination with DKK1LP may improve the clinical outcome of cancer patients when combined with chemotherapy or other standard therapies. DKK1LP-specific Th responses in MM patients may positively influence overall survival. The impact of DKK1-specific Th responses on clinical outcome will be evaluated in future studies.

In conclusion, DKK1LP provides a useful tool for propagation of both DKK1-specific Th1 cells and CTL, and may synergize with CTL-epitopes to enhance cancer cell killing. These findings provide a rationale for a clinical trial of DKK1LP-based immunotherapy against a broad spectrum of cancer types, as DKK1 is widely expressed by human cancer cells.

Disclosures
No conflicts of interest to disclose.

Contributions
JQ, RL, and QY initiated the study; JQ, RL and CZ designed the experiments and wrote the paper; RL and JQ performed most of the experiments and statistical analyses; QW prepared human samples; EB helped with animal experiments; MY assisted in generating mice; JH and WF provided important suggestions.

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