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Peptide Length Extension Skews the Minor HA-1 Antigen Presentation toward Activated Dendritic Cells but Reduces Its Presentation Efficiency

Lothar Hambach,* Zohara Aghai,* Jos Pool,* Nicolaus Kröger,† and Els Goulmy*

Minor histocompatibility Ags (mHags) are important targets of the graft-versus-leukemia effect after HLA-matched allogeneic stem cell transplantation. mHags are HLA-restricted polymorphic peptides expressed on normal and leukemia cells. Vaccination with hematopoiesis-restricted mHag peptides, such as HA-1, may boost the graft-versus-leukemia effect. However, some animal studies indicate that peptides exactly reflecting immunogenic T cell epitopes (short peptides [SPs]) induce tolerance that is potentially due to systemic Ag spreading. Peptide length extension (long peptides [LPs]) may optimize immune responses by restricting and prolonging Ag presentation on dendritic cells (DCs). In this study, we compared the in vitro characteristics and T cell-stimulatory capacities of a human 30-mer HA-1 LP with the 9-mer HA-1 SP. DCs presented the HA-1 LP and SP and expanded HA-1–specific cytotoxic T cell lines. As hypothesized, HA-1 LP presentation, but not SP presentation, was largely restricted to activated DCs and was nearly absent on other hematopoietic cells. However, DCs presented the HA-1 LP 2–3 log levels less efficiently than the SP. Finally, the decay of HA-1 LP and SP presentation on DCs was comparable. We conclude that HA-1 LP and SP differ in their in vitro characteristics and that only comparative clinical studies after allogeneic stem cell transplantation may reveal the optimal HA-1 vaccine. *The Journal of Immunology, 2010, 185: 000–000.

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Abbreviations used in this paper: Ad5E1, adenovirus type 5 early region 1; CD40L, CD40 ligand; DC, dendritic cell; GVHD, graft-versus-host disease; HLA, human leukocyte antigen; LP, long peptide; LPs, long peptide; mHag, minor histocompatibility Ag; SP, short peptide; TAA, tumor-associated Ag; UL2006-3482, project code for the Sander Foundation (Munich, Germany); and the Netherlands Organization for Scientific Research (Den Haag, The Netherlands).

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*Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands; †Department of Bone Marrow Transplantation, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.

Address correspondence and reprint requests to Dr. Lothar Hambach, Department of Immunohematology and Blood Transfusion, E3Q, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands. E-mail address: l.w.h.hambach@lumc.nl.

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Animal studies showed that C- and/or N-terminal extension of OVA-derived SPs with natural flanking sequences can improve the T cell response, particularly T cell priming (16, 17). The following arguments were attributed to the superiority of these so-called “long peptides” (LPs). First, LPs cannot bind directly to MHC molecules. Instead, they require processing before presentation of the immunogenic epitope. The superior Ag-processing capacity of professional APCs focuses the LP presentation on DCs. Second, restriction of OVA-derived LP presentation to the regional lymph nodes draining the coadministered adjuvants may guarantee Ag presentation on optimally activated APCs. Finally, in vivo presentation of OVA-derived LPs is prolonged compared with SPs as the result of a still-unknown mechanism (16).

HA-1 SP-based vaccination studies were initiated recently (K. van Besien and A. Ganser, personal communication). Meanwhile, the animal data described above prompted us to study the impact of HA-1 peptide extension on Ag presentation. We compared the in vitro characteristics of a selected HA-1 LP and SP with regard to the optimal requirements for their presentation by DCs, their presentation efficiency and persistency on DCs, and the peptide-presenting cell types in detail. We discuss the impact of our findings on HA-1 peptide vaccination after allogeneic SCT.

Materials and Methods

**Donor material**
Peripheral blood was collected from healthy donors. PBMCs were isolated by Ficoll density gradient centrifugation. Approval was obtained from the Leiden University Medical Center (LUMC) review board. Informed consent was provided according to the Declaration of Helsinki. HA-1 typing of donors was performed as previously described (18).

**Peptides**
Short 9-mer peptides and long 30-mer peptides were synthesized on an automated multiple-peptide synthesizer (Sryo; MultiSynTech, Witten, Germany), purified, and characterized by analytical reversed-phase HPLC. None of the mass spectrometry patterns of the LPs showed any signals for the SP. Peptides were dissolved at 25 μg/μl in 100% DMSO and stored at −20 °C. Peptides were further diluted to a concentration of 1 μg/μl in PBS and used in the assays in the indicated concentrations.

**Effector cells**
HLA-A2-restricted HA-1–specific CTL lines and the HA-1–specific CTL clone 1.7 were generated with PBMCs from healthy donors, as previously described (8), via weekly stimulation of PBMCs with autologous activated DCs pulsed with HA-1 peptide for 3 h. The percentage of HA-1–specific CTLs in the CTL lines was determined by staining with allophycocyanin-labeled tetramers, as described earlier (19).

**Stimulator cells**
HLA-A*0201**<sub>TM</sub>** EBV-transformed lymphoblastoid cell lines (LCLs) were generated in our laboratory, EBV LCLs and the TAP-deficient mutant cell line T2 were cultured in IMDM supplemented with 5% FCS. Fibroblasts (kind gift of Dr. Nicola Annels, Department of Pediatrics, LUMC, Leiden, The Netherlands) were cultured in IMDM supplemented with 5% FCS. Fibroblasts were harvested by thorough resuspension and washed with PBS and used in the assays in the indicated concentrations.

**Proliferation test**
The HA-1–specific CTL clone 1.7 was cultured for 3 d in 10% human serum in IMDM and 120 IU/ml IL-2 (Chiron, Amsterdam, The Netherlands). Before the proliferation assay, target cells were washed three times with PBS and resuspended in 10% human serum in IMDM. Responder T cells (1 × 10<sup>5</sup> cells/well) were cocultured with stimulator cells (2 × 10<sup>5</sup> cells/well) in 96-well flat-bottom microtiter plates for 24 h at 37 °C. Sixteen hours before harvesting, 0.5 Ci [<sup>3</sup>H]thymidine was added, and [<sup>3</sup>H]thymidine incorporation (cpm) was determined by liquid scintillation counting. The results are expressed as the mean of triplicate cultures.

**Chromium release assay**
In vitro cytotoxicity was measured in standard [51Cr]-release assays, as described earlier (8). In short, 2500 Cr<sup>51</sup>-labeled target cells were incubated with dilutions of effector CTLs for 4 h; supernatants were harvested for gamma counting using the following formula: percentage of specific lysis = (experimental release – spontaneous release)/(maximal release − spontaneous release) × 100%.

**Statistics**
Different groups were compared by a Mann–Whitney U test using SPSS 16.0 (SPSS, Chicago, IL). A p value <0.05 was considered statistically significant.

**Results**

**Selection of the HA-1 LP**
The length of the 30-aa HA-1 LP was chosen based on the capability of 25–35 mer LPs to induce HPV-specific immune responses in murine (16, 17, 21) and clinical studies (22, 23). Six HA-1 LPs of the HA-1 protein sequence containing the HA-1 epitope were selected (Fig. 1A). Only LPs with sequences flanking the N and C terminus of the epitope were used to maximize the demand for peptide processing before HA-1 epitope presentation. Subsequently, the LPs’ capacities to stimulate HA-1–specific CTL proliferation after peptide loading on CD40-activated monocyte-derived DCs were compared. Peptide loading was performed under serum-free conditions to minimize the risk for peptide degradation by serum proteases. All six HA-1 LPs induced HA-1–specific CTL proliferation to various extents, as determined by [<sup>3</sup>H]thymidine uptake. The HA-1 LP 123–152 induced the greatest level of HA-1–specific CTL proliferation (Fig. 1B). Therefore, HA-1 LP 123–152 was used in all subsequent experi-
ments and referred to as the HA-1 LP. The HA-1 nonameric peptide 137–145 comprising the exact HA-1 T cell epitope was referred to as HA-1 SP.

**HA-1 LP presentation is processing dependent**

The dose dependency of HA-1 LP presentation was tested by titrating HA-1 LP and SP to HLA-A2+/HA-1R EBV LCLs (derived from HLA-A2+ donors homozygous for the nonimmunogenic HA-1R allele) and TAP-deficient T2 cells and incubating for 3 h at 37˚C. HA-1 LP required 2–3 log levels more peptide than HA-1 SP to induce equal HA-1–specific CTL proliferation (Fig. 2A).

Next, the impact of the peptide incubation time and temperature on HA-1 LP and SP presentation was studied. The time dependency of HA-1 LP presentation was tested by incubation of HLA-A2+/HA-1RR EBV LCLs with HA-1 LP or SP for 1, 3, and 24 h at 37˚C. HA-1 LP presentation reached its maximum after 3 h (Fig. 2B), whereas presentation of HA-1 SP was comparable for all tested incubation times. The temperature dependency of HA-1 LP presentation was tested by incubation of HLA-A2+/HA-1RR EBV LCLs with HA-1 LP and SP at 4˚C and 37˚C. HA-1 LP presentation was largely abrogated at 4˚C, whereas SP was presented equally well at 4˚C and 37˚C (Fig. 2B).

Next, the relevance of cell-released enzymes for the presentation of HA-1 LPs was investigated. As described in detail below, T and B cells presented HA-1 SP but very little LP. Thus, we incubated these HA-1 LP nonpresenting T and B cells with HA-1 LP or SP in the presence of medium conditioned by LP-presenting cells (activated DCs or T2 cells). The presence of conditioned media was shown to enhance the presentation of HA-1 LPs by DCs but not by T and B cells.
medium did not result in HA-1 LP presentation by T and B cells (Fig. 2C). Thus, HA-1 LP presentation most likely results from cellular enzymatic processing and not from extracellular degradation.

**HA-1 LP presentation is proteasome and TAP independent**

Presentation of endogenous HA-1 in EBV LCLs and DCs from donors positive for the immunogenic HA-1H allele was inhibited by incubation with the proteasome inhibitors epoxomicin (Fig. 2D, upper panel) and lactacystin (Supplemental Fig. 1B). In contrast, incubation of DCs and EBV LCLs from donors homozygous for the nonimmunogenic HA-1R allele with proteasome inhibitors did not hamper HA-1 LP or SP presentation (Fig. 2D, lower panel). Thus, HA-1 LP presentation is, in contrast to endogenous HA-1 presentation, proteasome independent. Similar results were obtained for T2 cells and fibroblasts (Supplemental Fig. 1A, 1B). The TAP inhibitor ICP-47, which is capable of abrogating the presentation of endogenously expressed HA-1 in HA-1H EBV LCLs, did not reduce the presentation of HA-1 LP or SP (Fig. 2E). Combined with the HA-1 LP presentation by TAP-deficient T2 cells (Fig. 2A), these results suggest the TAP independence of HA-1 LP presentation.

**HA-1 LP is best presented by activated DCs**

The impact of DC activation on HA-1 peptide presentation was investigated. DCs were activated with CD40L trimers or tCD40Ls for 24 h and incubated with 1 μM HA-1 LP or SP for 3 h in 24-well plates. Flow cytometry revealed a marked upregulation of the costimulatory molecules CD80 and CD86 and of HLA-A2 after stimulation of PBMCs (Fig. 1A,1B). The TAP inhibitor ICP-47, which is capable of abrogating the presentation of endogenously expressed HA-1 in HA-1H EBV LCLs, did not reduce the presentation of HA-1 LP or SP (Fig. 2E). Combined with the HA-1 LP presentation by TAP-deficient T2 cells (Fig. 2A), these results suggest the TAP independence of HA-1 LP presentation.

**FIGURE 3.** Influence of DC activation on HA-1 LP presentation. A, HA-1–specific CTL proliferation stimulated by DCs from HA-1RR donors, activated or not for 24 h with CD40L trimers or tCD40Ls and loaded with 1 μM HA-1 LP (black bars) or SP (white bars) for 3 h. B, HA-1–specific CTL proliferation stimulated by nonactivated or activated HA-1RR DCs loaded with 1 μM HA-1 LP (black bars) and SP (white bars) for 3 or 24 h. DCs were activated with tCD40s 24 h earlier, simultaneously, or 6 h after peptide administration. Data are mean cpm of DCs from three (A) or four (B) donors; cpm was compared in a pairwise manner in different groups by the Mann–Whitney U test.

**FIGURE 4.** HA-1 presentation in different cell types. A, HA-1–specific CTL proliferation stimulated by activated DCs (○), T cells (△), and nonadherent monocytes (◇) from HA-1RR donors and by adherent fibroblasts (□) loaded with HA-1 LP (upper panel) or HA-1 SP (lower panel) for 3 h. Data are mean (± SD) cpm of three (DCs, T cells, B cells, and fibroblasts) or four (monocytes) experiments. B, HA-1–specific CTL proliferation in response to PBMCs (white bars), monocytes (black bars), B cells (gray bars) and T cells (hatched bars) from HA-1RR or HA-1H individuals. The target cells were not loaded or HA-1 SP loaded. Data are mean (± SD) cpm of three experiments.
The decays of HA-1 LP and SP peptide presentation on DCs are comparable

Next, persistence of HA-1 LP and SP presentation on activated DCs was compared. DCs loaded in 24-well plates with titrated amounts of peptides were placed in 96-well plates, and HA-1–specific CTLs or anti–HLA-A2–specific CTLs were added immediately (0 h) or after 24, 48, 72, 96, 120, 144, or 168 h. No change in proliferation of the anti–HLA-A2–specific CTL clone was observed when stimulated by HA-1 LP- and SP-loaded DCs, suggesting that the overall Ag-presenting capacity was stable. HA-1 LP and SP presentation declined after peptide loading of DCs, and the HA-1 LP and SP dose-response curves remained largely parallel throughout the observation period (Fig. 6, Supplemental Fig. 2A,B). Thus, the decays of HA-1 LP and SP presentation on DCs are comparable.

HA-1 LP presentation is skewed toward DCs

The capacity of activated DCs and other APCs to present HA-1 LP and SP was tested in peptide-titration experiments. T cells, B cells, and monocytes from HA-1RR donors were isolated from PBMCs. Purity of T cells, B cells, and monocytes was >90%, as determined by flow cytometry for CD3, CD19, and CD14 (data not shown). HA-1LP or HA-1SP was titrated to activated DCs, T cells, B cells, nonadherent monocytes, and adherent fibroblasts and incubated for 3 h. Subsequently, HA-1–specific CTL proliferation in response to peptide-loaded target cells was determined. Activated DCs required 2–3 log levels more HA-1 LP than SP to induce equal HA-1–specific CTL proliferation (Fig. 4A). Fibroblasts loaded with peptide under adherent conditions presented HA-1 LP and SP only slightly less efficiently than DCs (Fig. 4A). B cells and nonadherent monocytes induced little HA-1–specific CTL proliferation after loading with the maximal dose of 100 μM HA-1 LP (Fig. 4A, upper panel). HA-1 LP-loaded T cells did not induce HA-1–specific CTL proliferation (Fig. 4A, upper panel). In contrast, SP-loaded DCs, fibroblasts, T cells, B cells, and monocytes induced considerable HA-1–specific CTL proliferation (Fig. 4A, lower panel). Overall, HA-1 LP presentation is more restricted to DCs than HA-1 SP presentation. Interestingly, adherent monocytes and fibroblasts presented HA-1 LP better than nonadherent monocytes and fibroblasts (data not shown), suggesting that cell adherence may affect the extent of HA-1 LP presentation.

Hematopoietic cells of HA-1H individuals stimulate HA-1–specific CTL proliferation

Host chimeric cells frequently persist in patients after allogeneic HLA-matched SCT and may present the immunogenic host HA-1H allele to the transplanted HA-1H-negative donor-immune system. Therefore, we tested the capacity of T cells, B cells, and monocytes of an HA-1H individual to stimulate the proliferation of HA-1–specific CTLs. We found that, particularly, HA-1H naturally expressing monocytes effectively stimulate HA-1–specific CTL proliferation (Fig. 4B).

Discussion

Processing dependency of HA-1 LP presentation

Our data show that presentation of the selected HA-1 LP resulted from cellular enzymatic processing, because its presentation was time-, temperature-, and cell-type–dependent and not inducible via the supernatant of HA-1 LP-presenting cells. Presentation of HA-1 LP was 2–3 log levels less efficient than HA-1
SP, which is in accordance with previous reports on LPs derived from the TAAAs NY-ESO-1 LP (24), Melan-A, and gp100 (25). It is unclear whether insufficient Ag uptake or processing is responsible for the low presentation of LPs in HLA class I. Ags accessing the cytosol are usually processed via the conventional pathway of endogenous molecules. These Ags undergo proteasomal cleavage and are translocated by TAP to the endoplasmic reticulum, where they associate with HLA class I molecules before transport to the cell surface. Ags not accessing the cytosol are processed via alternative, typically proteasome- and/or TAP-independent pathways (26–28). Previous reports showed that TAA-derived LPs can be presented proteasome dependently [e.g., NY-ESO-1 (24) or Melan-A (25)] or independently [e.g., gp100 (25)] and TAP independently [e.g., NY-ESO-1 (24)]. In our study, HA-1 LP presentation was, irrespective of the cell type (DCs, EBV LCLs, T2 cells, and fibroblasts) and in contrast to the endogenous HA-1, not repressed by proteasome inhibitors. Moreover, the TAP inhibitor ICP47 did not repress HA-1 LP presentation by EBV LCLs. Thus, our results indicate that HA-1 LP is processed via an alternative pathway.

Potential vaccine characteristics of HA-1 LPs

We found that HA-1 LP presentation by DCs can be strongly increased via CD40 activation, which is described to enhance cross-presentation of soluble Ags (29). These data are in support of the concept that LPs favor presentation in the context of optimal costimulation (16, 17, 30). Nevertheless, the improved presentation of HA-1 LP by activated DCs is also surprising, because DC maturation downregulates endocytosis (31). However, similar observations were made previously for gp100 LP (but not Melan-A LP), which is best presented by LPs-matured DCs (25), and for exogenous OVA, which is best presented in MHC class I by murine DCs after maturation with TLR 3 and 9 agonists (32).

Further evaluation of the cell types presenting HA-1 peptides revealed that circulating T cells, B cells, and nonadherent monocytes hardly present HA-1 LP, whereas they effectively present HA-1 SP. This finding is in accordance with previous results showing that OVA-derived LPs are not presented by T cells or B cells isolated from local lymph nodes (16, 17). Consequently, HA-1 LP presentation is indeed skewed toward activated DCs. However, fibroblasts also presented HA-1 LP very well. Thus, HA-1 LPs can also be presented in the absence of optimal costimulation. This finding is important, because systemic spreading of peptides and subsequent presentation in a low-costimulatory context (e.g., in the lungs) was linked to the tolerance induction in the Ad5SE1 system in vivo (13–15). What determines the capacity of a cell to present HA-1 LPs remains unclear. Apart from the intrinsic Ag-presenting capacity of cells, circumstances like prolonged in vitro culture in the presence of growth factor-rich FCS (as for monocyte-derived DCs, EBV LCLs, and fibroblasts) or cell adherence (as for adherent monocytes and fibroblasts) might affect HA-1 LP presentation. These issues are subjects for further studies.

Finally, the superior vaccine efficacy of LPs over SPs has also been attributed to the, mechanistically unclear, longer persistence of LP presentation in vivo (16). DCs from draining lymph nodes very rapidly lose Ag presentation after isolation, which points toward an extracellular depot, rather than toward Ag storage within the DCs (16). However, a recent study also demonstrated slower decays of Melan-A and gp100 LP presentation compared with the respective SPs on the cellular level (25). In contrast, our study revealed that the decays of HA-1 LP and SP presentation are comparable. Remarkably, HA-1 SP was still detectable up to 7 d after DC loading with 100 μM peptide. This extremely long presentation might result from the high HLA-binding affinity of HA-1 and its low dissociation rate from HLA (33, 34). These features may stabilize HA-1 peptides better on DCs than TAA peptides, which frequently have a low HLA-binding affinity (35, 36). Overall, the phenomenon of prolonged LP presentation on the cellular level cannot be generalized and might differ between Ags of different HLA-binding affinity, as well as between peptide sequences. Future studies on HA-1 LPs may involve linkage of LPs to TLR agonists (37) or Abs (38) to facilitate receptor-mediated Ag uptake and to increase the extent and duration of HA-1 LP presentation.

**HA-1 LPs in the context of allogeneic SCT**

Activated DCs loaded with HA-1 LPs stimulated HA-1–specific CTL clones and effectively expanded polyclonal HA-1–specific CTL lines (from sensitized healthy donors), showing killing of leukemia cells in vitro. Notably, donor mHags emerging after allogeneic SCT are polyclonal memory T cells (5) already primed in the donor during pregnancy (39, 40) and/or in response to the patient’s mHags after allogeneic SCT. Earlier studies revealed a restricted TCR usage for recognition of HLA-A2/HA-1H by in vivo and in vitro HA-1 SP-induced HA-1–specific CTLs (41, 42). Interestingly, the HA-1 tetramer+ cells isolated from HA-1 LP-stimulated CTL lines also used the same TCR β variable chain TCRBV7-9 (Supplemental Fig. 3). These data suggest that HA-1 LPs might be promising vaccines to stimulate the in vivo pre-existing HA-1–specific CTLs capable of eradicating residual leukemia cells.

Yet, several questions regarding the superiority of HA-1 LPs over SPs as vaccines after allogeneic SCT remain. First, the low presentation efficiency of HA-1 LPs in vitro might be further aggravated in vivo because it is unknown when DCs, after allogeneic SCT, are functional enough to process and to present HA-1 LPs (43). Also, the general efficiency of alternative Ag-processing pathways, as shown for HA-1 LP in our study, in vivo is unknown (27). Second, our results show that HA-1 LP presentation is more restricted to activated DCs than HA-1 SP presentation, which may improve the quality of HA-1 LP-induced immune responses compared with SP-induced immune responses. However, in the potential HA-1 peptide vaccination strategy after allogeneic SCT, one needs to consider the transplanted donor hematopoiesis homozygous for the nonimmunogenic HA-1R allele and the host hematopoiesis positive for the immunogenic HA-1H allele. Depending on the conditioning, graft composition, and other factors, residual host chimeric cells frequently persist for many months after allogeneic SCT (44). Consequently, host-derived hematopoietic cells with less costimulatory capacity than DCs present HA-1H systemically. This assumption is underlined by our finding that endogenous HA-1H is well presented by circulating monocytes. Therefore, it seems questionable whether LP-mediated skewing of HA-1 peptide presentation to DCs may be beneficial over SPs in the allogeneic SCT setting. Finally, the reported greater efficacy of LPs in animal studies was particularly related to priming of naive T cells against neoantigens (16, 17, 21). However, mHag-specific immune responses after allogeneic SCT in humans are largely secondary immune responses. Therefore, the respective animal studies might not be predictive of the immunological situation after allogeneic SCT in humans.

In conclusion, careful balancing of the advantages and disadvantages of using HA-1 LPs or SPs as peptide vaccine does not provide clear guidance about which type of peptide may lead to optimal results after allogeneic SCT. Therefore, the optimal peptide for boosting HA-1–specific CTLs may only be determined in clinical trials directly comparing LPs with SPs.
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Disclosures
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