Chapter 8.

Peptide extension skews the HA-1 presentation towards activated dendritic cells but reduces the presentation efficiency

Peptide length extension skews the minor HA-1 antigen presentation towards activated dendritic cells but reduces its presentation efficiency

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Minor histocompatibility antigens (mHags) are important targets of the graft-versus-leukemia (GvL) effect after human leukocyte antigen (HLA)-matched allogeneic stem cell transplantation (SCT). MHags are HLA-restricted polymorphic peptides expressed on normal and leukemia cells. Vaccination with hematopoiesis-restricted mHag peptides like HA-1 may boost the GvL effect. However, some animal studies indicate that peptides exactly reflecting immunogenic T-cell epitopes ("short peptides", SPs) induce tolerance potentially due to systemic antigen spreading. Peptide length extension ("long peptides", LPs) may optimize immune responses by restricting and prolongating antigen presentation on dendritic cells (DCs). Here, 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 both 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 almost absent on other hematopoietic cells. However, DCs presented the HA-1 LP 2-3 log levels less efficiently than the SP. Finally, the decays of HA-1 LP and SP presentation on DCs were comparable. We conclude that HA-1 LP and SP differ in their in vitro characteristics and that only comparative clinical studies after allogeneic SCT may reveal the optimal HA-1 vaccine.

INTRODUCTION

The graft-versus-leukemia (GvL) effect and the graft-versus-host disease (GvHD) after HLA-matched stem cell transplantation (SCT) are mainly driven by allo-immune reactivities against minor histocompatibility antigens (mHags) (1). MHags are HLA-restricted polymorphic peptides derived from genes with differential tissue distribution (2). Whereas the ubiquitously expressed mHags, e.g. H-Y, are the prime in situ targets of GvHD (3), the hematopoiesis-restricted mHags might be used for boosting the GvL effect with low risk of GvHD (1). The mHag HA-1 is only expressed by human normal and malignant hematopoietic cells (2) and by solid tumors (4). The anti-tumor efficacy of HA-1 specific cytotoxic T lymphocytes (HA-1 CTLs) is demonstrated by two observations. First, the emergence of HA-1 (and HA-2) CTLs coincides with remissions of hematological malignancies after donor lymphocyte infusions (DLI) (5). Second, HA-1 CTLs significantly delay human leukemia progression (6) and destroy human breast cancer metastases in immunodeficient mice (7). The optimal clinical approach to target HA-1 is still unclear. In vitro generation of HA-1 CTLs for adoptive transfer is possible but too laborious for routine clinical practice (8). Alternatively, patients could be vaccinated with "off the shelf" HA-1 peptides to stimulate HA-1 CTLs emerging after allogeneic SCT. The HA-1 protein comprises two alleles resulting from a single amino acid (aa) polymorphism (Histidin (H) <-> Arginin (R)) (9). Only the HA-1^H (but not the HA-1^R) allele results in immunogenic T-cell epitopes (in HLA-A2 and –B60) (1,10). Thus, HLA-matched/HA-1 mismatched SCT results in strong T-cell

responses of the HA-1^{RR} donor against hematopoietic cells of the HA-1$^{HR\ or\ HH}$ patient. HA-1 vaccines to boost these responses are based on the immunogenic HA-1^{H} epitope.

Indications for the optimal design of HA-1 peptide vaccines can be deduced from therapeutic cancer vaccine studies in the autologous setting. Most studies are based on exact HLA class I binding peptides derived from the aa sequences of tumor-associated antigens (TAA) (11,12). These peptides reflect exactly the T-cell epitope of usually 9-10 aa and are subsequently named "short peptides" (SP). Clinical studies repetitively showed that vaccination with SPs can induce detectable immune responses (11,12). Nevertheless, the clinical responses to cancer peptide vaccination are still poor. Animal data showed that subcutaneous administration of SPs derived from the adenovirus type 5 early region 1 (Ad5E1) induces immunological tolerance (13-15), while vaccination with ex vivo Ad5E1 SP loaded dendritic cells (DCs) leads to immunity (15). The induction of tolerance by the subcutaneously administered Ad5E1 SPs was explained by systemic peptide spreading leading to peptide presentation by non-professional antigen presenting cells (APCs) far distant from the subcutaneous injection side, e.g. in the lung (13-15). Moreover, SPs can bind directly to MHC class I molecules of circulating APCs, e.g. B- and T-cells which are capable of peptide presentation in lymph nodes all over the body (16). Thus, systemic presentation of SPs in the absence of proper co-stimulatory molecules may cause suboptimal CTL responses or even tolerance.

Animal studies show that C- and/or N-terminal extension of ovalbumin (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 antigen 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 co-administered adjuvants may guarantee antigen presentation on optimally activated APCs. Finally, in vivo presentation of OVA-derived LPs is prolonged compared to SPs due to a still unknown mechanism (16).

HA-1 SP based vaccination studies have been recently initiated (K. van Besien, A. Ganser, personal communications). Meanwhile, the animal data described above prompted us to study the impact of HA-1 peptide extension on antigen presentation. In detail, we compared the in vitro characteristics of a selected HA-1 LP and the SP with regard to 1) the optimal requirements for their presentation by DCs, 2) their presentation efficiency and persistency on DCs and 3) the peptide presenting cell types. Finally, 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. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. Approval was obtained from the Leiden University Medical Center 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 peptide and long 30-mer peptides were synthesized on an automated multiple peptide synthesizer (Syro, MultiSynTech,Witten, Germany), purified and characterized by analytical reversed-phase high-performance liquid chromatography. None of the mass spectrometry patterns of the LPs showed any signals for the SP. Peptides were dissolved at 25 ug/ul in 100% dimethyl sulfoxid and stored at –20°C. Peptides were further diluted to a concentration of 1 ug/ul in PBS and used in the assays in the indicated concentrations.

Effector cells

HLA-A2 restricted HA-1 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 3h. 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^{pos} Epstein-Barr-virus (EBV)-transformed B lymphocytes (LCL) were generated in our lab. EBV LCLs and the TAP-deficient mutant cell line T2 were cultured in IMDM supplemented with 5% fetal calf serum (FCS). Fibroblasts (kind gifts of Dr. Nicola Annels, Dept. of Pediatrics, LUMC, Leiden, The Netherlands) were cultured in 10% FCS/IMDM. Mock-GFP and the TAP-inhibitor ICP-47-GFP (kindly provided by Dr. E. Wiertz, Dept. Medical Microbiology, LUMC, Leiden) transduced EBV LCLs have been described earlier (20). Monocyte derived dendritic cells were generated by isolation of CD14+ cells from PBMCs using CD14 Microbeads (Miltenyi Biotec, Utrecht, The Netherlands). The CD14+ cells were cultured in RPMI medium 1640 supplemented with 8% fetal calf serum, GM-CSF (Invitrogen, Breda, The Netherlands) 800 U/ml, IL-4 (Invitrogen) 250 U/ml. On day 2 and 4 medium was refreshed and immature dendritic cells were frozen on day 6. After thawing, dendritic cells were cultured in 24 well plate wells in the presence of GM-CSF and IL-4 for 24h. Subsequently, DCs were activated with 1 ug/ml CD40-ligand (CD40L) trimers (R&D Systems, Abingdon, UK) or with human CD40L transfected murine fibroblasts (tCD40L; kindly provided by Dr. C van Kooten, Department of Nephrology, Leiden University Medical Center). The fibroblasts were irradiated (90 Gy) and used at a concentration of $1x10^5$ cells / 24 well plate well. T-cells and B-cells were isolated from frozen CD14 depleted PBMCs by negative isolation to avoid activation of the cells (T-cell and B-cell isolation kit II, Miltenyi). Before the experiment, T-cells, B-cells and monocytes cells were cultured in 24 well plate wells in X-Vivo-15 (Lonza, Verviers, Belgium) for 24h to allow recovery from thawing.

Incubation with peptide and inhibitors

Stimulator cells were harvested by thorough re-suspension and washed with PBS. Peptide incubation of $5x10^5$ cells was performed in 500 ul X-vivo-15 or AimV (Gibco, Breda, The Netherlands; Figure 2B) at 37°C in 15 ml tubes unless stated otherwise. During incubation, tubes were placed horizontally to prevent cell accumulation at the tube bottom at high density. For subsequent assays, DC, monocytes, T-cells and B-cells were irradiated (30 Gy) to reduce the background proliferation of stimulator cells. HA-1^{RR} EBV LCLs, T2 cells and fibroblasts were fixed with glutaraldehyde, because irradiation was mostly not sufficient for these cells to suppress background proliferation. Before fixation, stimulator cells were washed three times with PBS. Fixation was performed with glutaraldehyde 0.05% (Sigma Aldrich, Zwijndrecht, The Netherlands) in PBS for 60 seconds. Reaction was stopped with 0.2M Glycine in PBS for 30 seconds and cells were washed three times with PBS. HA-1^{H} target cells were irradiated with 30 Gy (DCs) or 100 Gy (EBV LCLs) since glutaraldehyde fixation destroyed CTL recognition of the natural epitope. Proteasome dependency was tested by stimulator cell incubation with the proteasome inhibitors epoxomicin (20 and 2 μM) or lactacystin (40 and 4 μM) (Alexis biochemicals, Raamsdonksveer, The Netherlands) for 2h at 37°C. Subsequently, peptides were added and incubated for 3h.

Proliferation test

The HA-1 CTL clone 1.7 was cultured for 3 days in 10% human serum in IMDM and 120 IU/ml interleukin 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 ($1x10^4$ cells/well) were co-cultured with stimulator cells ($2x10^4$ cells/well) in 96-well flat-bottomed microtiter plates for 24 hours (Figure 2B: 72h) in 100 ul IMDM supplemented with 10% human serum. Sixteen hours before harvesting, 0.5 Ci of ^{3}H-thymidine was added. The ^{3}H-thymidine incorporation in counts per minute (c.p.m.) 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 ^{51}Cr release assays as described earlier (8). In short, 2500 ^{51}Cr-labeled target cells were incubated with dilutions of effector CTLs for 4 hours; supernatants were harvested for gamma counting: % specific lysis = (experimental release - spontaneous release)/(maximal release - spontaneous release) x 100%.

Statistics

Different groups were pair-wise compared by a Mann-Whitney-U test using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). A p-value < 0.05 was considered statistically significant.

RESULTS

Selection of the HA-1 long peptide

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 both murine (16,17,21) and in clinical studies (22,23). Six HA-1 LPs of the HA-1 protein sequence comprising the HA-1 epitope were selected (Figure 1A). Only LPs with sequences flanking both the N- or 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 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 of peptide degradation by serum proteases. All 6 HA-1 LPs induced HA-1 CTL proliferation to various extents as determined by ^{3}H thymidine uptake whereby the HA-1 LP 123-152 induced by trend the highest level of HA-1 CTL proliferation (Figure 1B). Therefore, HA-1 LP 123-152 was used in all subsequent experiments 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 the "HA-1 SP".

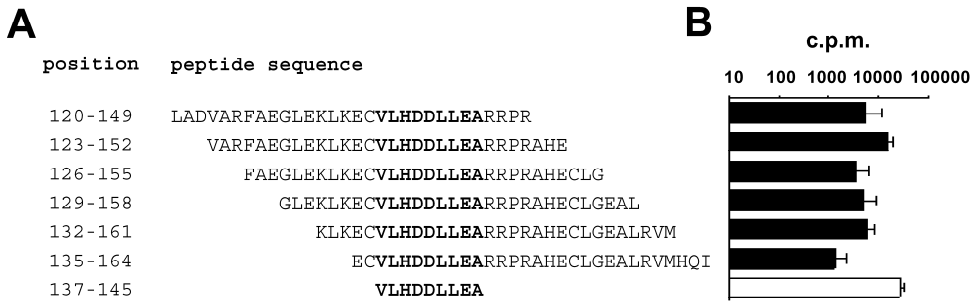

Figure 1. Presentation of HA-1 LPs by DCs. (A) Six different HA-1 LPs containing the immunogenic HLA-A2/HA-1^{H} epitope sequence (indicated in bold) and the SP were selected; (B) HA-1 CTL proliferation stimulated by DCs from donors homozygous for the non-immunogenic HA-1^{R} allele, loaded with LP (black bars) or SP (white bar) at 1 µM for 3h was tested by ^{3}H thymidine uptake. x-axis: mean scintillation in counts per minute (c.p.m.) of 3 experiments, error bars: standard deviation (SD).

HA-1 long peptide 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-1^{RR} EBV LCLs (derived from HLA-A2^{+} donors homozygous for the non-immunogenic HA-1^{R} allele) and TAP-deficient T2 cells and incubation for 3h at 37°C. HA-1 LP required 2-3 log levels more peptide than HA-1 SP to induce equal HA-1 CTL proliferation (Figure 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-1^{RR} EBV LCLs with HA-1 LP or SP for 1h, 3h and 24h at 37°C. Presentation of HA-1 LP reached its maximum after 3h (Figure 2B), while presentation of the 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-1^{RR} EBV LCLs with HA-1 LP and SP at 4°C and 37°C. HA-1 LP presentation was largely abrogated at 4°C, while SP was presented equally well at 4°C and 37°C (Figure 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 did present the HA-1 SP but hardly the LP. Thus, we incubated these HA-1 LP non-presenting T- and B-cells with HA-1 LP or SP in the presence of

medium "conditioned" for 24h by LP presenting cells (activated DCs or T2 cells). Presence of conditioned medium did not result in HA-1 LP presentation by T- and B-cells (Figure 2C). Thus, HA-1 LP presentation most likely results from cellular enzymatic processing and not from extracellular degradation.

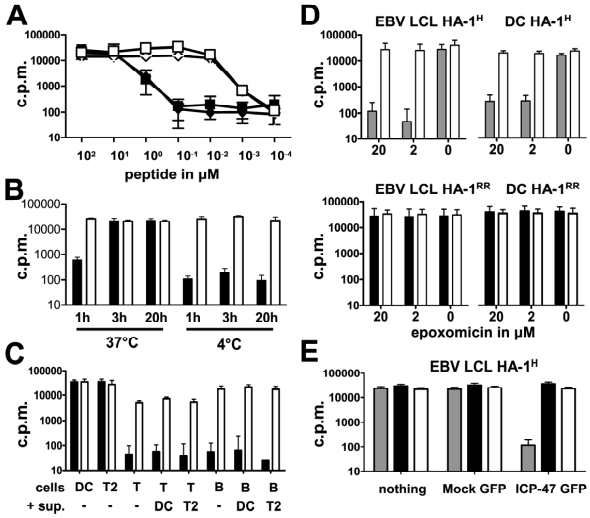

Figure 2. Processing-dependency of HA-1 LP. (A) HA-1 CTL proliferation stimulated by T2 cells (diamonds) and EBV LCLs (boxes) incubated with various HA-1 LP (filled symbols) or SP (open symbols) concentrations for 3h; x-axis: peptide concentration in µM; (B) HA-1 CTL proliferation stimulated by EBV LCLs incubated with HA-1 LP (black bars) and SP (white bars) at 37°C or 4°C for 1h, 3h or 20h. (C) HA-1 CTL proliferation after HA-1 LP (black bars) or SP (white bars) loading of DCs, T2 cells, T-cells, B-cells or T- and B-cells in the presence of 24h supernatant (sup.) from DCs or T2 cells; (D) HA-1 CTL proliferation stimulated by activated DCs and EBV LCLs from donors positive for the immunogenic HA-1^H allele (upper panel) or homozygous for the non-immunogenic HA-1^R allele (lower panel). Stimulator cells were treated with 20, 2 or 0 µM of the proteasome inhibitor epoxomicin. Subsequently, HA-1^H positive stimulator cells (upper panel) were not loaded (grey bars) or loaded with HA-1 SP (white bars); HA-1^{RR} stimulator cells (lower panel) were incubated with HA-1 LP (black bars) or SP (white bars); (E) HA-1 CTL proliferation stimulated by non-transduced, Mock-GFP or TAP inhibitor ICP-47-GFP transduced EBV LCLs from a donor positive for the immunogenic HA-1^H allele. The stimulator cells were not loaded (grey bars) or loaded with HA-1 LP (black bars) or SP (white bars); A-E: HA-1 LP 10 µM; HA-1 SP 10^{-1} µM (B, D-E) and 1 µM (C) ; y-axis: mean c.p.m. (background subtracted) of 3 experiments , error bars: SD.

HA-1 long peptide presentation is proteasome- and TAP-independent

Presentation of endogenous HA-1 in EBV LCLs and DCs from donors positive for the immunogenic HA-1^H allele was inhibited by incubation with the proteasome inhibitors epoxomicin (Figure 2D, upper panel) and lactacystin (Supplementary Figure S1B). In contrast, incubation of DCs and EBV LCLs from donors homozygous for the non-immunogenic HA-1^R allele with proteasome inhibitors did not hamper HA-1 LP or SP presentation (Figure 2D, lower panel). Thus, HA-1 LP presentation is – in contrast to endogeous HA-1 presentation - proteasome-independent. Similar results were obtained for T2 cells and fibroblasts (Supplementary Figure S1A, B). The TAP ("transporter associated with antigen processing") inhibitor ICP-47 capable of abrogating the presentation of endogenously expressed HA-1 in HA-1^H EBV LCLs did not reduce the presentation of HA-1 LP or SP (Figure 2E). Combined with the HA-1 LP presentation by TAP-deficient T2 cells (Figure 2A), these results suggest the TAP-independency of HA-1 LP presentation.

HA-1 long peptide 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 tCD40L fibroblasts for 24h and incubated with 1 µM HA-1 LP or SP for 3h in 24 well plates. Flow cytometry revealed a marked up-regulation of the co-stimulatory molecules CD80 and CD86 and of HLA-A2 after DC activation (data not shown). Both CD40L trimer and tCD40L fibroblast activated DCs induced a 4-6 times higher and statistically different (p=0.001 and p=0.004, respectively) level of HA-1 CTL proliferation than non-activated DCs (Figure 3A). Next, we tested whether HA-1 LP presentation can be further enhanced by prolonging the peptide incubation time or by changing the sequence of DC activation and peptide loading. tCD40L fibroblasts were added to non-activated DCs (group A) 24h before (group B), simultaneously to (group C) or 6h after (group D) addition of peptides. Again, activated DCs induced 3-5 times higher (A-B: p=0.009, A-C: p=0.003 and A-D: p=0.079) levels of HA-1 CTL proliferation compared to non-activated DCs. The magnitude of HA-1 CTL proliferation was not statistically different between the tested sequences of CD40 activation and peptide administration (B-C: p=0.631; C-D: p=0.497; B-D: p=0.661). Overall, DCs presented HA-1 LP by trend best after 3h peptide incubation subsequent to 24h DC activation (Figure 3B). Thus, DCs were peptide loaded for 3h after 24h activation in all subsequent experiments.

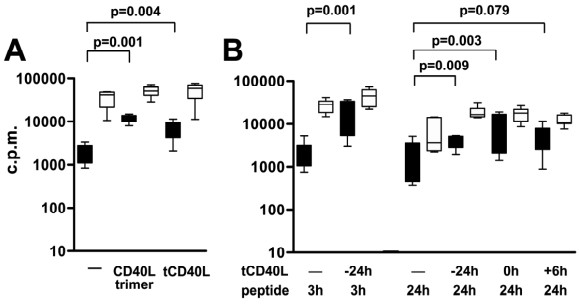

Figure 3. Influence of DC activation on HA-1 LP presentation. (A) HA-1 CTL proliferation stimulated by DCs from HA-1^{RR} donors, non-activated or activated for 24h with CD40L trimers or tCD40L fibroblasts and loaded with 1 µM HA-1 LP (black bars) or SP (white bars) for 3h. (B) HA-1 CTL proliferation stimulated by non-activated or activated HA-1^{RR} DCs loaded with 1 µM HA-1 LP (black bars) and SP (white bars) for 3h or 24h. DCs were activated with tCD40L fibroblasts 24h before, simultaneously or 6h after peptide administration. y-axis: mean c.p.m. of DCs from 3 different donors (A) or DCs from 4 different donors (B) depicted as Box-whisker plots. C.p.m. were pair-wise compared in different groups by Mann-Whitney-U test.

HA-1 long peptide pulsed DCs expand polyclonal HA-1 CTLs

Polyclonal HA-1 CTL lines were generated by weekly stimulations of PBLs from two healthy HA-1^{RR} blood donors with HA-1 LP and SP pulsed autologous DCs. Both donors were known to be immunized against HA-1^{H}. A dose of 10 µM for HA-1 LP was selected, since HA-1 CTL proliferation reached its plateau at this peptide dose (see Figure 6A). HA-1 SP was applied at 1 µM, i.e. the standard dose used in our protocols for HA-1 CTL generation (19). The percentage of HA-1 tetramer staining cells after 3 stimulations reached 7.2 and 9.8% for HA-1 LP and up to 51 and 3.1% for HA-1 SP stimulated CTL lines (Figure 4A). Expansion of total HA-1 tetramer staining cells subsequent to 4 stimulations was comparable for HA-1 LP and SP stimulated CTL lines (Figure 4B). The HA-1 LP and SP stimulated CTL lines from both donors lysed hematopoietic cells from HA-1^{H} individuals, including leukemia cells, but not from HA-1^{RR} individuals. Thus, both HA-1 LP and SP loaded DCs can expand high avidity polyclonal HA-1 CTLs.

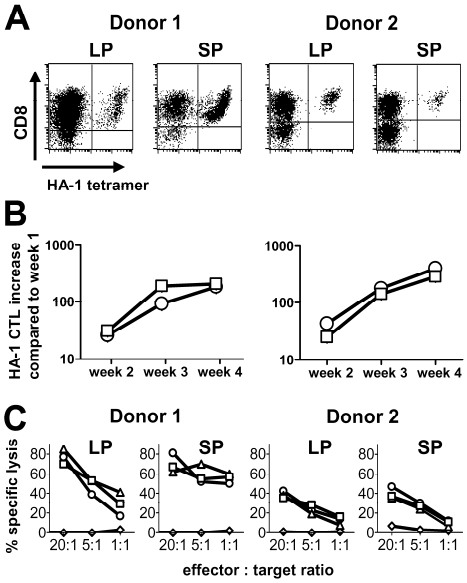

Figure 4. Expansion of HA-1 CTL lines with HA-1 LP or SP pulsed DCs. Polyclonal HA-1 CTL lines generated by weekly PBL stimulation with HA-1 LP or SP pulsed activated DCs from donors homozygous for the non-immunogenic HA-1^{R} allele; (A) HA-1 tetramer (x-axis) / CD8 (y-axis) staining of CTL lines from two different donors after 3 stimulations; (B) Increase of CD8/HA-1 tetramer positive cell numbers in HA-1 CTL lines subsequent to weekly stimulations with HA-1 LP (circles) or SP (boxes) loaded DCs; x-axis: weeks after first stimulation; y-axis: increase of HA-1 tetramer staining CTLs compared to day 7; (C) Cytotoxicity of the HA-1 CTL lines against HA-1^{H} EBV LCLs (boxes), HA-1^{H} leukemia cells (triangle), HA-1^{RR} EBV LCLs (diamonds) and HA-1 SP loaded HA-1^{RR} EBV LCLs (circles) determined in a ^{51}Cr release assay. X-axis: effector to target cell ratio; y-axis: % specific lysis.

The decays of HA-1 long and short 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 plate wells and HA-1 CTLs or anti-HLA-A2 CTLs were added directly (0h) or after 24h, 48, 72h, 96, 120h, 144h or 168h. No change in proliferation of the anti-HLA-A2 CTL clone was observed when stimulated by HA-1 LP and SP loaded DCs, suggesting that the overall antigen presenting capacity was stable. Both 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 (Figure 5, Supplementary Figure S2A and B). Thus, the decays of HA-1 LP and SP presentation on DCs are comparable.

HA-1 long peptide presentation is skewed towards 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, monocytes from HA-1^{RR} 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-1 LP or HA-1 SP were titrated to activated DCs, T-cells, B-cells, non-adherent monocytes and adherent fibroblasts and incubated for 3h. Subsequently, HA-1 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 CTL proliferation (Figure 6A). Fibroblasts peptide loaded under adherent conditions presented HA-1 LP and SP only slightly less efficiently than DCs (Figure 6A). B-cells and non-adherent monocytes induced only little HA-1 CTL proliferation after loading with the maximal dose of 100 μM HA-1 LP (Figure 6A, upper graph). HA-1 LP loaded T-cells did not induce HA-1 CTL proliferation (Figure 6A, upper graph). In contrast, SP loaded DCs, fibroblasts, T-cells, B-cells and monocytes induced considerable HA-1 CTL proliferation (Figure 6A, lower graph). 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 non-adherent monocytes and fibroblasts (data not shown) suggesting that cell adherence may affect the extent of HA-1 LP presentation.

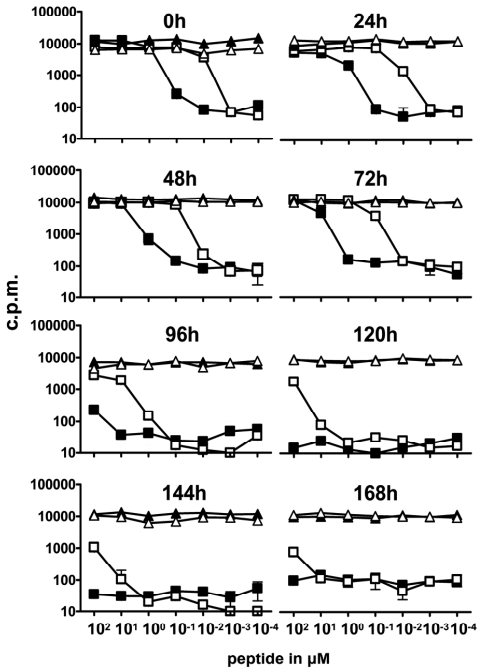

Figure 5. Persistence of HA-1 LP and SP presentation on DCs. CD40L-trimer activated HA-1^{RR} DCs were peptide loaded for 3h. HA-1 CTLs and anti-HLA-A2 CTLs were added after 0, 24, 48, 72, 96, 120, 148 and 168h. Subsequently, proliferation of HA-1 CTLs (boxes) and anti-HLA-A2 CTLs (triangles) in response to HA-1 LP (filled symbols) and SP (open symbols) loaded DCs was determined. x-axis: peptide concentration in µM; y-axis: mean c.p.m. of 3 replicates, error bars: SD.

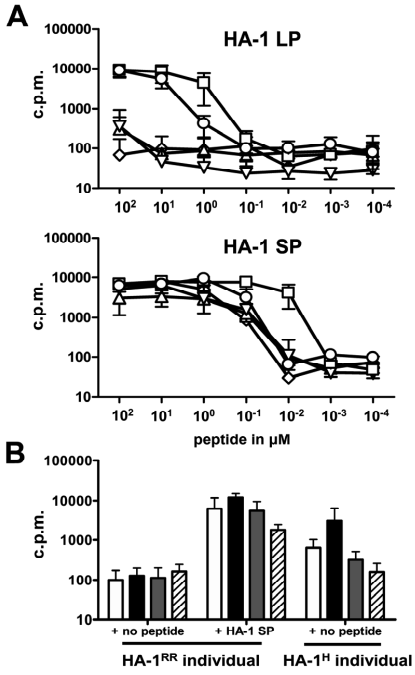

Figure 6. HA-1 presentation in different cell types. (A): HA-1 CTL proliferation stimulated by activated DCs (boxes), T-cells (diamonds), B-cells (up-triangles), non-adherent monocytes (down-triangles) from HA-1^{RR} donors and by adherent fibroblasts (circles) loaded with HA-1 LP (upper graph) or SP (lower graph) for 3h. x-axis: peptide concentration in µM; y-axis: mean c.p.m. of 3 (DCs, T-cells, B-cells and fibroblasts) - 4 (monocytes) experiments, error bars: SD. (B) HA-1 CTL proliferation in response to PBMCs (white bars), monocytes (black bars), B-cells (grey bars) and T-cells (hatched bars) from HA-1^{RR} or HA-1^{H} individuals. The target cells were not loaded or HA-1 SP loaded; y-axis: mean c.p.m. of 3 experiments, error bars: SD.

Hematopoietic cells of HA-1^{H} individuals stimulate HA-1 CTL proliferation

Host chimeric cells frequently persist in patients after allogeneic HLA-matched SCT and may present the immunogenic host HA-1^{H} allele to the transplanted HA-1^{H} negative donor immune system. Therefore, we tested the capacity of T-cells, B-cells and monocytes of an HA-1^{H} individual to stimulate the proliferation of HA-1 CTLs. We found that particularly HA-1^{H} naturally expressing monocytes effectively stimulate HA-1 CTL proliferation (Figure 6B).

DISCUSSION

Processing dependency of HA-1 long peptide presentation

Our data show that presentation of the selected HA-1 LP resulted from cellular enzymatic processing, since its presentation was time, temperature and cell-type dependent and not inducible via the supernatant of HA-1 LP presenting cells. Presentation of the HA-1 LP was 2-3 log levels less efficient than the SP, which is in accordance with previous reports on LPs derived from the TAAs NY-ESO-1 LP (24), Melan A and gp100 (25). It is unclear, whether insufficient antigen uptake or processing is responsible for the low presentation of LPs in HLA-class I. Antigens accessing the cytosol are usually processed via the conventional pathway of endogenous molecules. These antigens undergo proteasomal cleavage and are TAP-translocated to the endoplasmic reticulum where they associate with HLA class I molecules before transport to the cell surface. Antigens 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 MelanA (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.

HA-1 long peptides in view of their potential vaccine characteristics

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 antigens (29). These data are in support of the concept that LPs favor presentation in the context of optimal co-stimulation (16,17,30). Nevertheless, the improved presentation of HA-1 LP by activated DCs is also surprising, since DC maturation down-regulates endocytosis (31). However, similar observations have been made earlier for gp100 LP (but not MelanA LP) which is best presented by lipopolysaccharide matured DCs (25) and for exogenous OVA which is best presented in MHC class I by murine DCs after maturation with Toll-like receptor 3 and 9 agonists (32).

Further evaluation of the cell types presenting HA-1 peptides revealed that circulating T-cells, B-cells and non-adherent monocytes hardly present HA-1 LP while they effectively present the 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 towards activated DCs. However, also fibroblasts presented HA-1 LP very well. Thus, HA-1 LPs can also be presented in the absence of optimal co-stimulation. This finding is important, since systemic spreading of peptides and subsequent presentation in a low co-stimulatory context e.g. in the lungs was linked to the tolerance induction in the Ad5E1 system in vivo (13-15). It remains unclear, what determines the capacity of a cell to present HA-1 LPs. Apart from the intrinsic antigen presenting capacity of cells, circumstances like prolonged in vitro culture in the presence of growth-factor-rich fetal calf serum (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

loose antigen presentation after isolation which points towards an extracellular depot rather than antigen storage within the DCs (16). However, a recent study also demonstrated slower decays of MelanA and gp100 LP presentation compared to the respective SPs on cellular level (25). In contrast, our study revealed that the decays of HA-1 LP and SP presentation are comparable. Remarkably, the HA-1 SP was still detectable up to 7 days after DC loading with 100 uM 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 cellular level cannot be generalized and might differ between antigens of different HLA binding affinity and perhaps also between peptide sequences. Future studies on HA-1 LPs may involve linkage of LPs to TLR agonists (37) or antibodies (38) to facilitate receptor-mediated antigen uptake and to increase extent and duration of HA-1 LP presentation.

HA-1 long peptides in the context of allogeneic SCT

Activated DCs loaded with HA-1 LPs not only stimulated HA-1 CTL clones, but also effectively expanded polyclonal HA-1 CTL lines (from sensitized healthy donors) showing killing of leukemia cells in vitro. Notably, donor mHag CTLs emerging after allogeneic SCT are polyclonal memory T-cells (5) already primed either 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 the HLA2/HA-1 specific ligand by in vivo and by in vitro HA-1 SP induced HA-1 specific CTLs (41,42). Interestingly, also the HA-1 tetramer positive cells isolated from HA-1 LP stimulated CTL lines used the same T-cell receptor β variable chain TCRBV7-9 (Supplementary Figure S3). These data suggest that HA-1 LPs might be indeed promising vaccines to stimulate the in vivo pre-exisiting HA-1 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 antigen 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 compared to SP induced immune responses. However, in the potential HA-1 peptide vaccination strategy after allogeneic SCT one needs to consider both the transplanted donor-hematopoiesis homozygous for the non-immunogenic HA-1^R allele and the host-hematopoiesis positive for the immunogenic HA-1^H.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 co-stimulatory capacity than DCs present HA-1^H systemically. This assumption is underlined by our finding that endogenous HA-1^H is well presented by circulating monocytes. Therefore, it appears 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 higher efficacy of LPs in animal studies was particularly related to priming of naïve T-cells against neo-antigens (16,17,21). However, mHag specific immune responses after allogeneic SCT in man are (as discussed above) largely secondary immune responses. Therefore, the respective animal studies might not be predictive for the immunological situation after allogeneic SCT in man.

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

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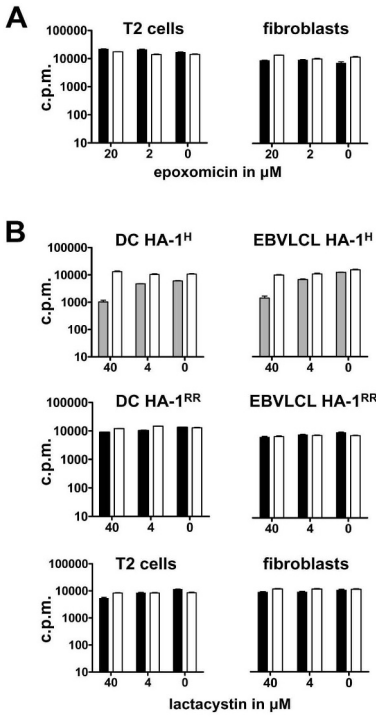
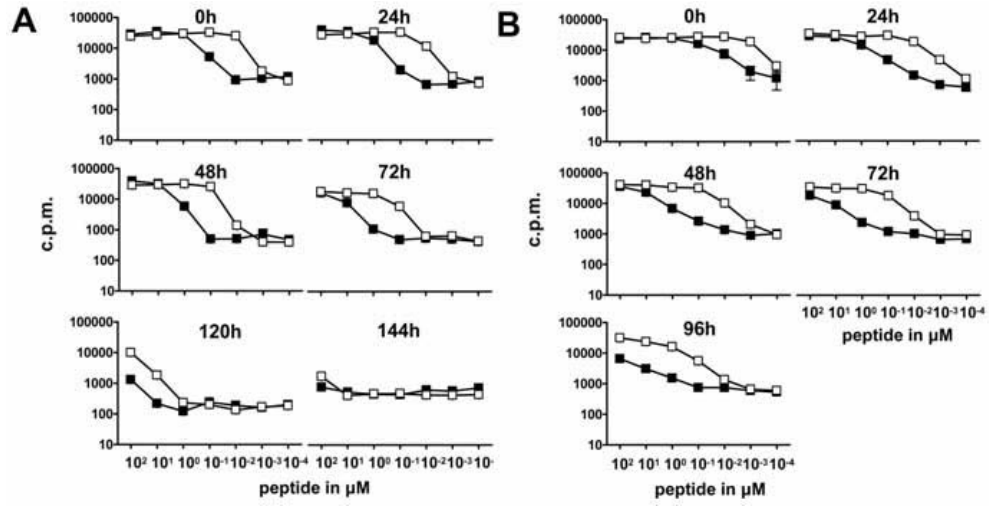
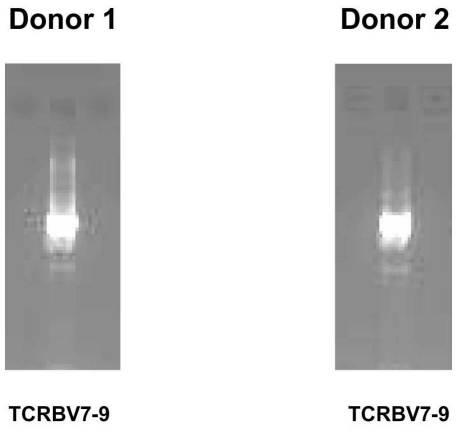
SUPPLEMENTARY FIGURES



Supplementary Figure S1. Treatment of target cells with proteasome inhibitors. A-B. HA-1 CTL proliferation after incubation with activated DCs and EBV LCLs from HA-1H or HA-1RR donors, T2 cells and fibroblasts. Stimulator cells were treated with the proteasome inhibitor epoxomicin (A) or lactacystin (B); B: black bars: HA-1 LP 10 µM loaded; white bars: HA-1 SP 10-1 µM loaded; grey bars: endogenously expressed HA-1H; y-axis: mean c.p.m. (background subtracted) of 3 replicates, error bars: SD.



Supplementary Figure S2. Persistence of HA-1 LP and SP presentation on DCs. CD40L activated DCs were peptide loaded for 3h. HA-1 CTLs were added to 10.000 (A) or 20.000 (B) DCs per well after 0-144h. Subsequently, HA-1 CTL proliferation in response to HA-1 LP (filled boxes) and SP (open boxes) loaded DCs was determined. x-axis: peptide concentration in µM; y-axis: c.p.m., error bars: SD.



Supplementary Figure S3. TCBV family specific PCR. HA-1 tetramer staining cells were isolated from both HA-1 LP peptide stimulated HA-1 CTL lines by flow cytometry, RNA was isolated and cDNA was amplified by polymerase chain reaction (PCR) using 32 TCRBV family specific forward primers and one common reverse primer TTCTCTTGACCATGGCCATC. The PCR resulted in one very prominent signal for TCRBV7- (amplified with the forward primer CAATTTCTGAACACAACCG; naming in accordance with the new IMGT nomenclature) that co-existed with few other but largely weaker TCRBV bands (the latter are not shown).