Enhanced Cross-Presentation and Improved CD8+ T Cell Responses after Mannosylation of Synthetic Long Peptides in Mice

Judith Rauen1, Christoph Kreer1, Arlette Paillard1, Suzanne van Duikeren2, Willemien E. Benchkuijsen2, Marcel G. Camps2, A. Rob P. M. Valentijn3a, Ferry Ossendorp2, Jan W. Drijfhout2, Ramon Arens2*, Sven Burgdorf1,2*,

1 Life and Medical Sciences Institute, University of Bonn, Bonn, Germany, 2 Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands, 3 Department of Bio-organic Synthesis, Leiden University Medical Center, Leiden, the Netherlands

Abstract

The use of synthetic long peptides (SLP) has been proven to be a promising approach to induce adaptive immune responses in vaccination strategies. Here, we analyzed whether the efficiency to activate cytotoxic T cells by SLP-based vaccinations can be increased by conjugating SLPs to mannose residues. We could demonstrate that mannosylation of SLPs results in increased internalization by the mannose receptor (MR) on murine antigen-presenting cells. MR-mediated internalization targeted the mannosylated SLPs into early endosomes, from where they were cross-presented very efficiently compared to non-mannosylated SLPs. The influence of SLP mannosylation was specific for cross-presentation, as no influence on MHC II-restricted presentation was observed. Additionally, we showed that vaccination of mice with mannosylated SLPs containing epitopes from either ovalbumin or HPV E7 resulted in enhanced proliferation and activation of antigen-specific CD8+ T cells. These findings demonstrate that mannosylation of SLPs augments the induction of a cytotoxic T cell response in vitro and in vivo and might be a promising approach to induce cytotoxic T cell responses in e.g. cancer therapy and anti-viral immunity.
increasing cross-presentation of mannosylated peptides

Materials and Methods

Mice

C57BL/6, MR−/−, OT-I and OT-II mice were maintained in the central animal facility of the LUMC. All mice were housed in specific pathogen-free conditions and used at 8–10 weeks of age. All animal experiments were approved by the Animal Experiments Committee leading to better clinical success [5].

A very effective approach to improving the uptake and/or activation of APCs is the use of mannoprotected SLPs. The acetyl protecting group on the mannos side chains was removed using Tesser’s base. Peptides were analyzed using UPLC-MS and MALDI-ToF mass spectrometry. Alexa647 modified peptides were prepared from the corresponding cysteine-containing precursor peptides. The cysteine-containing peptides were treated overnight with an excess of AlexaFlour647 C2-maleimide (A20347, Molecular Probes) in a mixture of DMSO and a sodiumphosphate buffer at pH 7.6–8.3. The Alexa647 labeled peptides obtained were subsequently purified using reversed phase chromatography using a C18 column. The structure of the labeled peptides was ensured using MALDI-ToF mass spectrometry. Purity of the generated peptides was >80%.

Flow cytometric analysis of peptide uptake

BM-DCs were incubated with 250 nM Alexa Fluor 647-labeled SLPs or 250 ng/ml Alexa Fluor 647-labeled OVA for 15 min at 37°C. Cells were harvested, washed and antigen uptake monitored by flow cytometry. Mean fluorescence intensity was calculated using FlowJo software.

Intracellular distribution of SLPs by immunofluorescence microscopy

Cells were pulsed for 15 min with 200 nM fluorochrome-labeled SLPs and chased for another 20 min with medium. Staining experiments were performed as described before [17]. For co-localizing experiments with transferrin, OVA or Lucifer Yellow (LY), fluorochrome-labeled SLPs were incubated together with 5 μg/ml fluorochrome-labeled transferrin, 250 ng/ml fluorochrome-labeled OVA or 0.3 mg/ml lucifer yellow. Nuclei were visualized with 1 μg/ml of the DNA-intercalating dye 4,6-diamidino-2-phenylindole (DAPI). Cells were analyzed with an ApoTome microscope (Zeiss). Co-localization was quantified by calculating the Pearson correlation coefficient and Mander’s overlap coefficient using the ImageJ software.

Proliferation of antigen-specific T cells

BM-DCs from wildtype or MR-deficient mice were incubated with 200 nM mannosylated or non-mannosylated SLPs or 0.5 mg/ml OVA for 2 h and co-cultivated with CFSE-labeled OT-I or OT-II T cells (labeled 15 min with 2 μM CFSE) for 3 days. Proliferation was assessed by flow cytometric analysis of the CFSE dilution. Division index and percentage dividing cells were calculated using the FlowJo software.

Peptide immunization and detection of in vivo T cell expansion

Mannosylated or non-mannosylated SLPs were dissolved in PBS. Per mouse, 75 μg SLPs were injected subcutaneously (s.c.) or intradermally (i.d.) in a total volume of 200 μl or 50 μl, respectively, with CpG as adjuvant (ODN1826, type B; 20 μg per mouse; purchased from InvivoGen). At day 7 post-vaccination, cell surface staining was performed on freshly prepared peripheral blood mononuclear cells and splenocytes after red blood cell lysis. Cells were surface stained for 30 min with allophycocyanin-labeled OVA257–264 or E749–57 tetramers and fluorescently labeled antibodies specific to mouse CD3 and CD8 in staining buffer (PBS containing 1% FCS and 0.05% sodium azide). 7-AAD (Life Technologies) was used for dead cell exclusion. Flow cytometric intracellular cytokine analysis of peripheral blood mononuclear cells and splenocytes was performed after 5 h stimulation with the short HPV16 E749–57 peptide (5 μg/ml) in presence of Brefeldin A.
were purchased from eBioscience and BD Pharmingen were generated according to standard procedures. Fluorescently Mannosylation targets SLPs towards early endosomes

Results

Antibodies and MHC class I tetramers

Rat anti-MR (MR5D5) and rat anti-CD86 (PO3) were from AbD Serotec; rat anti-LAMP-1 (1D4B) from BD; rabbit anti-EEA1 (H-300) from Santa Cruz; rabbit anti-LY (A5751) from Life Technologies; rat anti-CD6 (53-6.7), rat anti-CD40 (1C10), mouse anti-MHC I (28-14-8) and hamster anti-CD80 (16-10A1) were from eBioscience and all secondary antibodies from Life Technologies; rat anti-MR (MR5D3) and rat anti-CD86 (PO3) were from AbD Serotec; rat anti-LAMP-1 (1D4B) from BD; rabbit anti-EEA1 (H-300) from Santa Cruz; rabbit anti-LY (A5751) from Life Technologies; rat anti-CD6 (53-6.7), rat anti-CD40 (1C10), mouse anti-MHC I (28-14-8) and hamster anti-CD80 (16-10A1) were from eBioscience and all secondary antibodies from Life Technologies. H-2Kb OVA257–264 and H-2Db E749–57, tetramers were generated according to standard procedures. Fluorescently labeled antibodies against mouse CD3, CD8, IFN-γ and TNFα were purchased from eBioscience and BD Pharmingen.

Statistical analysis

P values were calculated by two-tailed T test (n≥3) using Excel (Microsoft) or Prism (Graphpad) Software.

Increased MR-mediated uptake of mannosylated SLPs

To investigate whether mannosylation of SLPs influenced their presentation and the ensuing T cell activation, we synthesized different mannosylated and non-mannosylated peptides (Fig. 1). We generated mannosylated and non-mannosylated peptides containing the MHC I-restricted epitope (peptides 1 and 2 in Fig. 1) or the MHC II-restricted epitope (peptides 3 and 4 in Fig. 1) of OVA. To monitor antigen uptake and routing, we additionally conjugated mannosylated or non-mannosylated peptides to the fluorochrome Alexa647 (peptides 5 and 6 in Fig. 1).

First, we analyzed whether mannosylation resulted in altered endocytic properties of the SLPs. To this end, we incubated bone marrow-derived DCs (BM-DCs) with fluorochrome-labeled SLPs and analyzed uptake by immunofluorescence microscopy (Fig. 2A) and flow cytometry (Fig. 2B, 2C, S1). Whereas no significant uptake of non-mannosylated peptides could be observed, we detected a clear internalization of mannosylated SLPs. This uptake was comparable to the internalization of OVA, which is highly mannosylated in its natural form [21], indicating that mannosylation of SLPs results in enhanced uptake. The specific increase in uptake of mannosylated peptides was not observed using BM-DCs from MR-deficient mice (Fig. 2A, 2B, 2C, S1), demonstrating that enhanced uptake was due to MR-mediated endocytosis.

Mannosylation targets SLPs towards early endosomes

Since the MR targets internalized antigens towards an early endosomal compartment, from where efficient cross-presentation takes place, we questioned whether mannosylation might target SLPs towards the same endocytic compartment. Therefore, we first incubated DCs simultaneously with fluorochrome-labeled SLPs and OVA, which in BM-DCs is targeted by the MR into these early endosomes [17], and analyzed their intracellular distribution by immunofluorescence microscopy. Figure 3A demonstrates a clear co-localization of mannosylated SLPs and OVA, indicating that mannosylation might indeed target SLPs towards early endosomes. To verify this hypothesis, we analyzed the co-localization of mannosylated peptides with different endosome markers. For OVA, a clear co-localization of mannosylated SLPs with the MR itself and with the early endosome markers early endosome antigen 1 (EEA1) and transferrin (Trf) was observed, whereas the lysosomal marker Lysosomal-associated membrane protein 1 (LAMP1) and the pinocytosis marker lucifer yellow (LY), which upon internalization is rapidly targeted towards lysosomes, do not co-localize with mannosylated SLPs (Fig. 3B). These observations demonstrate that increased uptake of mannosylated SLPs by the MR indeed targets them towards early endosomes.

Increased cross-presentation but not MHC II-restricted presentation of mannosylated SLPs

Since antigens targeted towards early endosomes by the MR are efficiently processed for cross-presentation [17], we examined whether increased MR-mediated endocytosis and targeting towards early endosomes might also enhance cross-presentation of mannosylated SLPs. Therefore, we treated wild-type or MR-deficient DCs with mannosylated or non-mannosylated SLPs containing the MHC I-restricted epitope OVA257–264. Subsequently, we incubated the DCs with CFSE-labeled OVA-specific T cells (OT-I) and monitored T cell proliferation by flow cytometry. In wild-type DCs, poor T cell proliferation was observed after DC treatment with non-mannosylated SLPs (Fig. 4A, quantification in Fig. 4B). After treatment with mannosylated SLPs, however, a strong T cell proliferation could be monitored. This proliferation was comparable to samples where...
induced a strong up-regulation of CD40, CD80, CD86 and MHC I, the addition of SLPs did not alter the expression of these molecules, regardless whether SLPs were mannosylated or not (Fig. 4C), pointing out that the addition of mannosylated SLPs did not alter DC maturation. To fully exclude any effects of mannosylated ligands on the overall capacities of DCs to activate T cells, we pre-treated DCs with mannosylated or non-mannosylated SLPs containing the MHC II-restricted epitope of OVA (peptides 3 and 4 in figure 1). Afterwards, we loaded these DCs with a non-mannosylated (short) peptide, containing only the MHC I-restricted epitope SIINFEKL, and analyzed proliferation of OT-I T cells. Importantly, no effect on OT-I T cell proliferation was observed after pre-treatment with mannosylated SLPs (Fig. 4D), demonstrating that the overall capacities of DCs to activate T cells is not influenced by the addition of mannosylated SLPs.

Next, we investigated the effect of SLP mannosylation on MHC II-restricted presentation. To this end, we incubated wildtype or MR-deficient DCs with mannosylated or non-mannosylated SLPs containing the MHC II-restricted epitope OVA253-261. Subsequent incubation with CFSE-labeled OVA-specific CD4+ T cells (OT-II) revealed that mannosylation of SLPs did not influence MHC II-restricted presentation (Fig. 4E, quantification in Fig. 4F). Consistently, the presence of the MR did not affect MHC II-restricted presentation of (mannosylated or non-mannosylated) SLPs, demonstrating that the enhancing effect of mannosylation is restricted to antigen cross-presentation and confirming again that the overall capacities of DCs to activate T cells remain unaltered by the addition of mannosylated SLPs.

**Increased proliferation of CD8+ T cells in vivo after treatment with mannosylated SLPs**

Finally, we aimed at investigating the effect of mannosylating SLPs on cross-presentation in vivo. Therefore, we first injected mice subcutaneously (s.c.) or intradermally (i.d.) with mannosylated or non-mannosylated SLPs containing the MHC I-restricted OVA epitope [peptides 1 and 2 in Fig. 1] and used CpG as an adjuvant. After 7 days, we used MHC class I tetramer staining to monitor the frequency of antigen-specific T cells in the blood and spleen. Whereas no differences in T cell responses could be observed between s.c. injection of mannosylated versus non-mannosylated SLPs, i.d. injection of mannosylated SLPs induced pronounced CD8+ T cell responses compared to non-mannosylated SLPs in both the blood and the spleen (Fig. 5A and 5B). These results point out that mannosylation of SLPs indeed can enhance cross-presentation of SLPs, especially if they are injected intradermally.

To investigate whether these differences also hold true for other antigens, we used mannosylated and non-mannosylated SLPs containing a HPV16 E7 epitope. As for SLPs containing OVA epitopes, we injected these peptides s.c. or i.d. into recipient mice and monitored CD8+ T cell expansion. Consistently to the results obtained for the SLPs containing the OVA epitope, mannosylated SLPs bearing the HPV epitope elucidated an enhanced antigen-specific T cell expansion compared to non-mannosylated SLPs in both blood and spleen (Fig. 5C and 5D). These differences were most pronounced if the SLPs were i.d. injected. Differences in T cell numbers were also observed after immunization in the absence of CpG, demonstrating that they were not due to a differential effect of CpG (Fig. S2).

Additional to an increased expansion, T cells activated after injection of mannosylated SLPs depicted an increased expression of IFN-γ and TNFα compared to non-mannosylated SLPs.
Discussion

In order to be cross-presented efficiently, SLPs need to be internalized and processed within the DC [22]. Since the stability of loaded MHC I molecules at the cell membrane is limited [23] but migration of the DCs towards the draining lymph nodes for T cell activation is a time-consuming process, it is essential that continuous cross-presentation occurs to supply the DC with freshly loaded MHC I molecules. Such persistent cross-presentation depends on prolonged antigen stability, since prompt antigen degradation by endo/lysosomal proteases rapidly destroys putative MHC I epitopes [24,25]. Therefore, it has been proposed that for efficient cross-presentation, antigens are targeted towards specific antigen storage compartments, where they are protected from rapid degradation by lysosomal proteases and from where continuous processing can take place [25]. Since the MR specifically targets its ligands towards a non-degradative subset of early endosomes [17,26,27], from where processing for cross-presentation efficiently can take place [18,19], the MR seems to be an optimal target for vaccination purposes aimed at the induction of a cytotoxic CD8+ T cell response [28,29].

In this study, we demonstrated that mannosylation of SLPs increased their MR-mediated uptake, resulting in enhanced cross-presentation and CD8+ T cell activation both in vitro and in vivo. Increased cross-presentation was not due to an altered overall capacity of the DC to activate T cells caused by mannosylated ligands, as the expression of co-stimulatory molecules remained unaffected and the addition of an irrelevant mannosylated SLP did not alter proliferation of OT-I cells. Interestingly, increased CD8+ T cell activation was most pronounced after injecting the SLPs i.d., whereas s.c. injection of mannosylated SLPs only lead to a marginal difference compared to non-mannosylated SLPs. These differences most likely can be explained by a differential MR
Figure 4. Cross-presentation and MHC II-restricted presentation of mannosylated SLPs. A) Wildtype or MR-deficient BM-DCs were incubated with OVA or SLPs and incubated with CFSE-labeled OT-I T cells. After 3 days, T cell proliferation was determined by flow cytometry after gating on CD8⁺ cells. B) Quantification of A), depicting the division index and the percentage of dividing OT-I T cells. C) BM-DCs were treated with 100 ng/ml LPS or 50 nM mannosylated or non-mannosylated SLPs. After 24 h, expression of CD40, CD80, CD86 and MHC I were analyzed by flow cytometry. Control: Isotype control. D) BM-DCs were pre-treated with 50 nM mannosylated or non-mannosylated SLPs containing the MHC II epitope of OVA for 1 h before they were loaded with the SIINFEKL short peptide for another 3 h and incubated with CFSE-labeled OT-I T cells. After 3 days, T
cell proliferation was determined by flow cytometry after gating on CD8\(^+\) cells. E) Wildtype or MR-deficient BM-DCs were incubated with OVA or SLPs and incubated with CFSE-labeled OT-II T cells. After 3 days, T cell proliferation was determined by flow cytometry after gating on CD4\(^+\) cells. F) Quantification of E), depicting the division index and the percentage of dividing OT-II cells. Depicted are representative results of at least 3 independent experiments.

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expression in the differential parts of the skin. Whereas dermal DCs express high levels of MR [20], no MR expression is observed in Langerhans cells of the epidermis [30].

In accordance with increased cross-presentation and activation of cytotoxic T cells by mannosylated SLPs observed here, targeting of the breast cancer mucin MUC1 towards the MR resulted in enhanced cross-presentation, an increased CD8\(^+\) T cell cytotoxicity and a reduced humoral response.

In a previous study, we demonstrated that in murine BM-DCs low amounts of antigens internalized by fluid phase pinocytosis were targeted rapidly towards lysosomes, where they co-localized with and were presented very efficiently on MHC II molecules [17]. In contrast to pinocytosed antigens, MR-internalized antigens were not targeted towards MHC II\(^+\) compartments and did not contribute to MHC II-restricted antigen presentation [17]. Similarly, in this study, low amounts of non-mannosylated SLPs

**Figure 5. In vivo T cell activation by mannosylated SLPs.** A) Mice were injected i.d. with 75 \(\mu\)g of OVA-specific mannosylated or non-mannosylated SLPs and 20 \(\mu\)g Cpg. After 7 days, antigen-specific T cells in the blood were monitored by flow cytometry using epitope-specific tetramers. Cells were gated for CD8. B) Quantitative analysis of epitope-specific T cells in the blood or in the spleen after s.c or i.d. injection of SLPs. Graphs show percentage of tetramer-positive CD8\(^+\) T cells. C) as in A) using HPV-specific SLPs. D) Quantitative analysis of epitope-specific T cells in the blood or in the spleen after s.c or i.d. injection of HPV-specific SLPs. E) Intracellular cytokine staining of splenic CD8\(^+\) T cells after i.d. injection of HPV-specific mannosylated or non-mannosylated peptides and Cpg as above. F) Quantitative analysis of intracellular cytokines in T cells isolated from the spleen after s.c or i.d. injection of HPV-specific SLPs. Graphs show percentage of IFN\(\gamma\)\(^{+}\) TNF\(\alpha\)\(^{+}\) cells amongst all CD8\(^+\) T cells. Dot plots depict representative results of 2 independent experiments. Bar graphs depict pooled results of 2 independent experiments (n = 9–10).

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were presented very efficiently on MHC II, whereas increased uptake after mannosylation specifically enhanced MR-mediated cross-presentation without influencing MHC II-restricted presentation, confirming the close correlation of the MR with cross-presentation. This correlation might also explain the limited success of MR-directed antigens in animal models like experimental autoimmune encephalomyelitis (EAE) [31,32,33], which is known to be mediated mainly by CD4+ T cells. Nevertheless, some studies also report of increased MHC II-restricted antigen presentation of MR-internalized antigens [34,35]. In these studies, the authors conjugated their antigens to MR-specific antibodies. However, cross-linking of the MR by the use of MR-specific antibodies influences DC maturation [36] and might also alter antigen routing and presentation as shown for dectin-1, another C-type lectin receptor. Whereas monovalent dectin-1 ligands are targeted towards early endosomes for cross-presentation, polyvalent ligands are rapidly targeted towards lysosomes, resulting in increased MHC II-restricted presentation [37]. These findings point out that receptor cross-linking regulates intracellular routing and presentation of internalized antigens and might be an alternative explanation for the observed MHC II-restricted presentation resulting from antibody-mediated MR-targeting. Similarly, increased MHC II-restricted presentation of mannosylated peptides or mannosylated BSA by human monocyte-derived DCs has been observed [38,39]. These cells, however, express additional C-type lectin receptors like DC-SIGN [40], which is not expressed on murine BM-DCs. Since DC-SIGN also binds and internalizes mannosylated ligands [40,41] and DC-SIGN-mediated endocytosis leads to efficient presentation on MHC II [40], it is feasible that MHC II presentation observed in these studies has been the result of antigen internalization via DC-SIGN.

Taken together, we could demonstrate that mannosylation of SLPS causes increased MR-mediated uptake and antigen routing into early endosomes, resulting in efficient cross-presentation and enhanced CD8+ T cell activation in vivo and in vitro. These findings might help to optimize vaccination strategies aimed at the induction of a cytotoxic T cell response in e.g. anti-tumor and anti-viral therapies.

Supporting Information

Figure S1 Uptake of mannosylated or non-mannosylated SLP in time course experiment. Wildtype or MR-deficient BM-DCs were incubated with 200 nM mannosylated SLPS or non-mannosylated SLPS for the indicated time points. Antigen uptake was monitored by flow cytometry (gated on all living cells).

(DOCX)

Figure S2 Immunization with mannosylated or non-mannosylated SLP in the absence of CpG. Mice were injected i.d. with 75 µg of HPV-specific mannosylated or non-mannosylated SLPS in the absence of CpG. After 7 days, antigen-specific T cells in the blood were monitored by flow cytometry using epitope-specific tetramers. Bars show percentage of tetramer+ T cells.

(DOCX)

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Author Contributions

Conceived and designed the experiments: RA SB. FO. Performed the experiments: JR CK AP SvD WEB MGC. Analyzed the data: JR AP SvD WEB RA SB. Contributed reagents/materials/analysis tools: JWD ARPMV. Contributed to the writing of the manuscript: RA SB.

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