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# Research Article

# The translocation motif of hepatitis B virus improves protein vaccination

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**Abstract.** Cell-penetrating peptides (CPPs) have been shown to improve antigen loading of dendritic cell vaccines. Here we asked whether fusion of a CPP to a protein improves its immunogenicity when this fusion protein is directly applied as vaccine. We used the cell-penetrating translocation motif (TLM) derived from the hepatitis B virus, because no size limitation of cargos has been observed. Increased immunogenicity was observed when TLM was fused to ovalbumin (TLM-ova). TLM-ova was found to be superior to ova in inducing proliferation and cytotoxicity of ova-specific CD8+ T cells *in vitro* and *in vivo*. Using ovalbumin-expressing thymoma cells (EG7-ova), an improved anti-tumor immune response was observed for TLM-ova vaccination versus vaccination with ova. Moreover, TLM-ova vaccination induced a higher titer of anti-ovalbumin IgG2a antibodies compared to ova. These data demonstrate that CPP-protein vaccines can improve cellular as well as humoral immune responses.

**Keywords.** Cell penetrating peptide, translocation motif of hepatitis B virus, tumor immunity, ovalbumin, protein vaccination.

During the last decade, several proteins and peptides have been characterized that can penetrate cellular membranes and enter the cell, delivering their cargo molecules into the cytoplasm and/or nucleus. These motifs are derived e.g. from the third helix of the antennapedia homeodomain, the human immunodeficiency virus protein Tat, the Kaposi fibroblast growth factor and the herpes simplex virus type-1 structural protein VP22 [1–4]. Cell-penetrating peptides (CPP) may be internalized into cells by mechanisms that require no energy and that are receptor independent [5]. Different models, e.g. the inverted micelle model, the pore formation model and the carpet model, have been suggested [6–8]. However, more recently, for some of these peptides, evidence has been presented that internalization is mediated by endocytotic processes [9–11].

Previously, an amphiphatic alpha-helical structure 12 amino acids in length (PLSSIFSRIGDP) in the PreS2 domain of the human hepatitis B virus (HBV) surface antigens was identified which mediates cell permeability. This sequence has been termed the translocation motif (TLM) [12]. Integrity of the TLM is crucial for the infectivity of HBV [our unpublished observations]. The TLM translocates in an energy-, receptor- and temperature-independent diffusion process across the membrane into the cytoplasm of cells without affecting cell integrity [12]. Cell permeability can be achieved if the TLM is fused to peptides or proteins [13–15]. In this study, we

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analyzed whether the TLM might also improve immunization with protein antigens.

Induction of T-cell-mediated immune responses requires MHC class-I- and II-dependent presentation of defined peptides on antigen-presenting cells (APCs) to CD8+ and CD4+ T cells, respectively. While CD8+ T lymphocytes (CTLs) are usually effector cells that directly kill their target cells by cytotoxic molecules, CD4+ T cells are usually helper cells that regulate immune responses. MHC class I molecules present peptide fragments of cytosolic and nuclear proteins, while the bulk of MHC class-II-complexed peptides are degradation products of exogenous proteins [16]. Several strategies have been described for directly loading antigens into the MHC class I presentation pathway of APCs with varying success [17–19]. One approach for antigen transfer into APCs, e.g. dendritic cells (DCs), is the use of the CPP. These molecules can deliver cargos fused or associated to them into the cytoplasm of cells and therefore make them directly accessible to the MHC class I presentation pathway [20–22]. APCs loaded with cell permeable peptides have been successfully used *in vitro* and *in vivo* for induction of CD8+ T-cell-mediated immune responses [21–26]. However, at present there is little information available whether CPP can be used to improve the immunogenicity and efficacy of complete proteins when CPP fusion proteins are administered directly as vaccines [27]. In this study we analyzed whether the TLM improves immunogenicity of proteins used for immunization. Ovalbumin (ova) served as a well-characterized model antigen in this study, enabling the analysis of CD8+ T cell responses *in vitro* and *in vivo*, using T cell receptor (TCR) transgenic mice (OT-I mice) [28]. The vaccine efficacy of TLM-ova fusion proteins was tested *in vivo* in a mouse tumor model (EG7-ova thymoma) [29]. The effect of TLM-ova on the humoral response was analyzed by ELISA.

## Materials and methods

**Mice and tumor cell lines.** For the tumor experiments, C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). OT-I mice (CD8+ TCR transgenic mice) recognize the ova-derived cognate peptide SIINFEKL [28] and were bred at the animal facility of the Max-Delbrueck-Center (Berlin, Germany). C57BL/6 and OT-I mice used for the *in vitro* and adoptive transfer experiments were provided by the laboratory animal facility of the Federal Institute for Risk Assessment (Berlin, Germany).

EL-4 thymoma cells and OVA-transfected EL-4 cells (EG7-ova) [29] were obtained from the American Type Culture Collection (Manassas, Va.) and cultured in DMEM containing 10% FCS.

**Synthetic peptides.** The SIINFEKL peptide (ovalbumin, aa 257–264) was purchased from EMC (Tübingen, Germany). The unrelated H-2K$^b$-binding peptide RGYVYQGL (vesicular stomatitis virus nucleoprotein, aa 52–59) was kindly provided by Dr. S. Stevanovic (University of Tübingen, Germany).

**Construction of ova-plasmids and production of recombinant proteins.** The coding sequence for the ova construct was amplified by PCR using primers which elongate the full-length ova cDNA from plasmid pAC-neo-OVAI (kindly provided by Prof. Rammensee, University of Tübingen, Germany) for the additional sequence of the hemagglutinin-tag (HA-tag) at the 3′-end. The coding sequence of the TLM-ova construct was amplified similarly as before except for the sequence encoding the PreS2-TLM that was introduced by a forward primer upstream of the coding sequence for the full-length ova cDNA. The PCR products were ligated in frame to a 5′-coding sequence for an N-terminal $(His)_6$-tag into the vector peT15b (Merck Biosciences, Bad Soden, Germany).

Forward primer for the ova construct: 5′-GGGAATTC-CATATGGGCTCCATCGGTGCAG-3′; forward primer for the TLM-ova construct: 5′-GGGAATTCCATATGCC-CTTATCGTCAATCTTCTCGAGGATTGGGGACCCTG GCTCCATCGTGCAGC-3′; reverse primer for the construct ova and TLM-ova: 5′-CGCGGATCCAGCATAGT-CTGGGACGTCATATGGATAAGGGGAAACACATCT-GCC-3′.

Recombinant ovalbumin proteins were produced in *Escherichia coli* BL21(D3). The proteins were purified by affinity chromatography on $Ni^{2+}$-NTA-agarose and subsequent anion exchange chromatography [30] under native conditions. Proteins were analyzed after SDS-PAGE (12% gels) by Coomassie staining. For Western blot analysis, proteins were transferred to a PVDF membrane (Amersham Pharmacia Biosciences, Freiburg, Germany). After blocking, the membrane was stained with an anti-HA-tag antibody (clone 12CA5; Roche, Mannheim, Germany) as primary antibody and with horseradish-peroxidase-conjugated goat anti-mouse IgG, (Amersham Pharmacia Biosciences) as secondary antibody. The blot was developed with enhanced chemiluminescence (Amersham Pharmacia Biosciences Freiburg, Germany). Eluted protein fractions of high purity were pooled, dialyzed against PBS and sterile filtered. The control protein TLM-eGFP (TLM fused to the enhanced green fluorescent protein) has been described previously [12]. The purification of TLM-eGFP was carried out as for ova proteins, but because of its high purity, no anion exchange chromatography was performed.

**Fluorescence microscopy.** Cells were incubated with the respective recombinant ovalbumin proteins and washed with cold PBS three times. Cells were then fixed in 4% paraformaldeyde in PBS (20 min, room temperature) and permeabilized with 0.05% Tween 20 in PBS. For indirect

immunofluorescence staining, a primary antibody directed against the HA-tag (clone 12CA5; Roche) and Cy3-conjugated goat anti-mouse IgG (Dianova, Hamburg, Germany) were used. Stained cells were analyzed using an immunofluorescence microscope (Leica, Wetzlar, Germany).

**Flow cytometry.** For flow cytometric analysis, the following antibodies were used: PE-labeled anti-mouse CD69 (clone H1.2F3), PE-labeled anti-mouse interferon (IFN)-$\gamma$ (clone XMG1.2), FITC-labeled anti-mouse V$\alpha$2 TCR (clone B20.1) (all from BD Biosciences, Heidelberg, Germany) and Cy5-labeled anti-mouse CD8 (clone 53-6.72). Anti-CD16/CD32 (clone 2.4G2, BD Biosciences) was used to block Fc receptors. Dead cells were excluded by propidium iodide staining. For intracellular cytokine staining, cells were fixed and permeabilized using standard protocols. Cells were analyzed using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences).

**Labeling of cells with 5-6-carboxyfluorescein diacetate succinimidyl ester.** Splenocytes were labeled with 5 µM 5-6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, Karslruhe, Germany) in PBS containing 0.1% BSA for 15 min at room temperature. After labeling, cells were washed twice with PBS/BSA and twice with PBS.

***In vivo* cytotoxicitiy assay.** To prepare target cells for *in vivo* evaluation of cytotoxic activity, splenocytes of C57BL/6 mice were prepared and erythrocytes removed. The cells were divided into three fractions. Fraction 1 was pulsed with 10 µM SIINFEKL peptide for 90 min at 37 °C, washed twice and labeled with 5 µM CFSE (high) as described above. Fraction 2 was pulsed with 10 µM unrelated RGYVYQGL peptide and labeled with 0.5 µM CFSE (intermediate). Fraction 3 was not incubated with peptide but labeled with 0.05 µM CFSE (low). For adoptive transfer, $3\times10^7$ total cells ($1\times10^7$ cells of each population) were injected intravenously (i.v.), mice were sacrified 16 h after adoptive transfer, splenocytes were prepared and the percentage of the three differentially labeled fluorescent target populations was analyzed by flow cytometry. The ratio r between the percentages of RGYVYQGL-pulsed versus SIINFEKL-pulsed cells was calculated to obtain a numerical value of cytotoxicity.

**Stimulation of OT-I splenocytes *in vitro* with recombinant ovalbumin proteins.** Splenocytes of OT-I mice were prepared and erythrocytes removed using standard protocols. Splenocytes were resuspended in T cell medium (RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, 1 mM Na pyruvate, 2 mM L-glutamine, 50 µM 2-mercaptoethanol and non-essential amino acids). OT-I splenocytes $5–6\times10^6$ were cultured in a volume of 2 ml (37 °C, 6% $CO_2$). Increasing concentrations of recombinant ovalbumin proteins were titrated into the cultures (0.1–2.5 µM protein). Cells without peptide or cells either incubated with 100 nM RGYVYQGL peptide or with 1 µM TLM-eGFP protein served as negative controls. As a positive control, cells were stimulated with 25 nM SIINFEKL peptide, a peptide concentration that led to optimal proliferation in previous experiments.
At different time points, T cell activation was assessed by the mean fluorescent intensity (MFI) of CD69 expression or IFN-$\gamma$ production of specific CD8+ V$\alpha$2+ T cells by flow cytometry. Proliferation was measured as CFSE dilution by flow cytometry.

**Vaccination of C57BL/6 mice with ovalbumin proteins after adoptive transfer of OT-I splenocytes.** Total OT-I splenocytes (containing $5\times10^6$ CD8+ V$\alpha$2+ T cells) were adoptively transferred in C57BL/6 mice. For the proliferation assay, splenocytes were labeled with CFSE as described above. Eighteen hours after adoptive transfer, mice were immunized intraperitoneally (i.p.) with recombinant ovalbumin proteins (10 µg protein per mouse for proliferation assay; 125 µg protein per mouse for cytotoxicity assay). To determine proliferation of OT-I T cells, mice were sacrificed 48–52 h after immunization. Splenocytes were prepared and proliferation of CD8+ V$\alpha$2+ T cells in the spleen was determined by flow cytometry. To investigate the cytotoxic activity of specific CD8+ T cells, an *in vivo* cytotoxic assay was performed 7 days after immunization as described above. Mice that received OT-I splenocytes, but were not immunized, served as a negative control.

**Immunization and tumor challenge experiments.** For tumor protection experiments, C57BL/6 mice or OT-I mice were immunized subcutaneously (s.c.) in the right flanks with 100 µg recombinant ovalbumin proteins plus CpG-ODN ('immuneasy'; Qiagen, Hilden, Germany) as adjuvant. Control groups remained untreated or were immunized with adjuvant only. Fourteen days after immunization, mice were challenged s.c. in the left flanks with $5\times10^5$ EG7-ova cells. Tumor size was determined biweekly for the following 60 days. Animals bearing tumors of $15\times15\times15$ mm$^3$ were scored as tumor positive and euthanized.

**Determination of ovalbumin-specific antibodies.** Ovalbumin-specific IgG2a serum levels were measured by ELISA. Serum was incubated on ova-coated (20 µg/ml; Sigma, Deisenhofen, Germany) microtiter plates, IgG2a was detected with a specific biotinylated monoclonal antibody (BD Biosciences), followed by streptavidin peroxidase (Calbiochem-Novabiochem, Bad Soden, Germany). The reaction was developed with tetramethylbenzidine

(TMB) (Fluka, Neu-Ulm, Germany), stopped with 1M sulfuric acid, and plates were measured at a wavelength of 450/490 nm. The anti-ova IgG2a antibody titers of the samples were related to pooled standard serum generated from sensitized BALB/c mice, with a specific activity arbitrarily set at 2000 units/ml (U/ml).

## Results

**Generation of cell-permeable ovalbumin proteins.** Expression vectors encoding the TLM-containing ova protein (TLM-ova) or the wild-type ova protein (ova) as a fusion protein with an N-terminal hexa-his-tag and a C-terminal HA-tag were constructed (Fig. 1a). The recombinant ovalbumin proteins were produced in *E. coli* BL21 (D3) and purified by sequential $Ni^{2+}$-NTA-agarose chromatography followed by anion exchange chromatography under native conditions [30]. Purified proteins were separated by SDS-PAGE and analyzed by Coomassie staining (Fig. 1b). As shown in Figure 1b, recombinant ovalbumin proteins of high purity were isolated. The Western blot analysis using an HA-tag- specific antibody that recognizes the C terminus of the fusion proteins confirmed that full-length proteins were indeed isolated by the affinity chromatography (Fig. 1c).

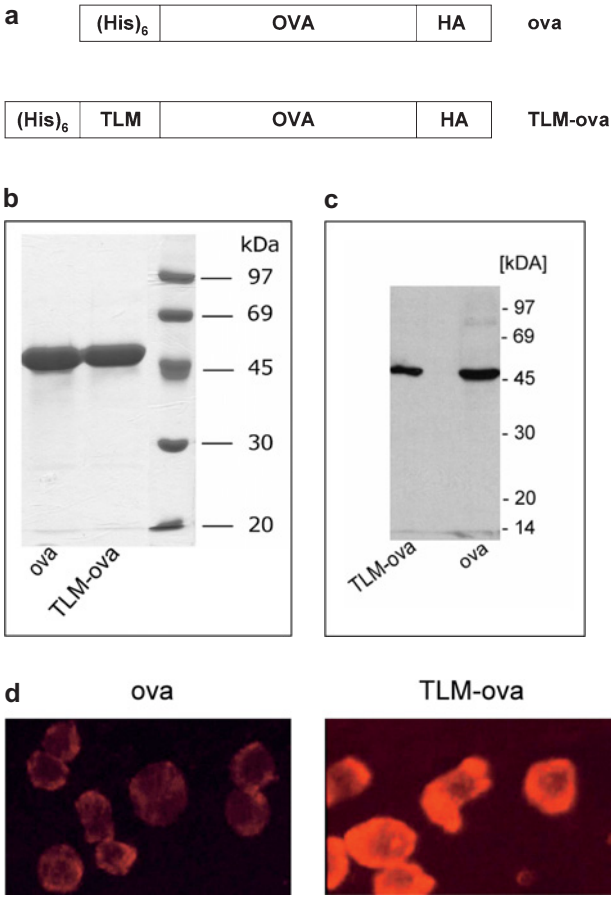



**Figure 1.** Recombinant ovalbumin proteins. (*a*) Schematic representation of recombinant ovalbumin proteins (*b, c*) Purification of recombinant ovalbumin proteins produced in *E. coli*; proteins were separated on 12% SDS-PAGE gels and detected by Coomassie staining (*b*) or by Western blotting using an HA-tag-specific antibody (*c*). (*d*) TLM-ova displays cell permeability in EL-4 thymoma cells. EL-4 cells were incubated with recombinant ovalbumin proteins (2 µM, 16 h), washed, fixed, permeabilized and stained with anti-HA-tag mAb and Cy3-conjugated goat anti-mouse IgG. Stained cells were analyzed by fluorescence microscopy.

To analyze whether fusion of the TLM to ovalbumin generates cell-permeable fusion proteins, mouse thymoma cells (EL-4 cells) were grown in the presence of TLM-ova or ova alone. Immunofluorescence microscopy demonstrated that only cells incubated with TLM-ova clearly showed specific staining of the cytoplasm, while those incubated with ova showed only background staining (Fig. 1d). This indicates that fusion of the TLM to ovalbumin generates a cell-permeable fusion protein.

**Improved *in vitro* stimulation of OT-I splenocytes with TLM-ova.** To determine the capacity of the respective recombinant ova-proteins to stimulate ova-specific TCR-transgenic CD8+ T cells (OT-I T cells), splenocytes of OT-I mice were pulsed *in vitro* with recombinant TLM-ova or ova proteins. TLM-ova caused a stronger activation, cytokine secretion and proliferation of OT-I T cells, as demonstrated by higher expression of the activation marker CD69, increased production of IFN-$\gamma$ and stronger proliferation of specific CD8+ T cells *in vitro*, compared to cultures pulsed with ova (Fig. 2a–c). To exclude any unspecific activation of OT-I T cells, splenocytes were also incubated with either an irrelevant H-2$K^b$-binding peptide or an irrelevant TLM-carrying protein (TLM-eGFP). These controls induced only marginal activation of specific CD8+ T cells (shown is a representative example for IFN-$\gamma$ production, Fig. 2b), excluding the possibility that the TLM per se exerts any effects. These data indicate that the cell-permeable ova fusion protein induces a stronger stimulation of OT-I splenocytes than the corresponding non-cell-permeable ova protein.

**Enhanced proliferation and cytotoxic activity of OT-I T cells after immunization with TLM-ova *in vivo.*** The next set of experiments was performed to analyze whether TLM-ova also improves activation (proliferation or cytotoxic activity) of OT-I T cells *in vivo.* This was tested by adoptively transferring OT-I splenocytes into C57BL/6 mice followed by immunization with either TLM-ova or ova. To asses proliferation, $5\times10^6$ CFSE-labeled OT-I T cells were transferred followed by i.p. immunization of these mice with the respective recombinant ova protein. Proliferation of OT-I T cells reisolated from the spleen 52 h after immunization was evaluated by flow cytometric analysis. As shown in Figure 3, at low doses of

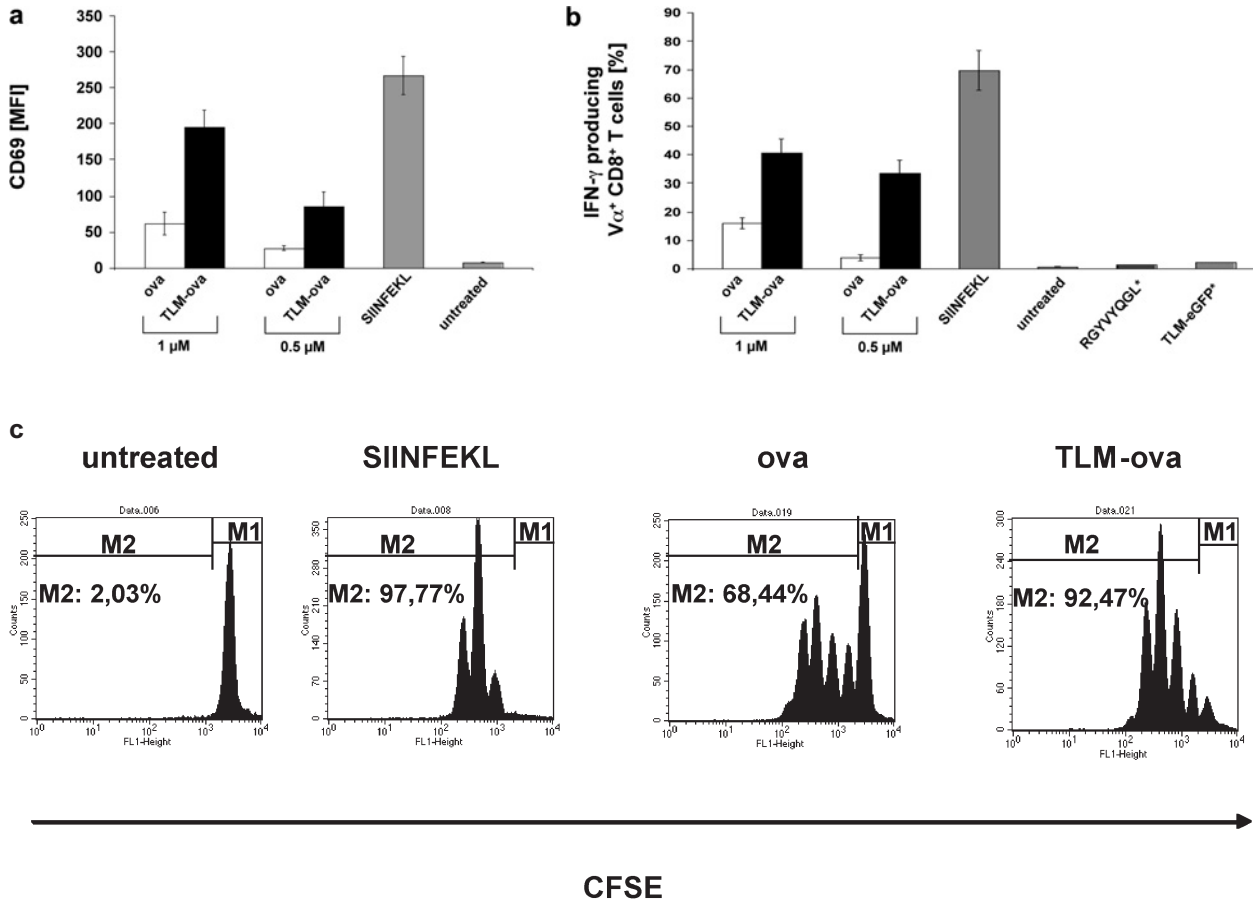



**Figure 2.** Improved *in vitro* stimulation of OT-I splenocytes with TLM-ova. (*a, b*) Splenocytes of OT-I mice were cultured with recombinant ovalbumin proteins (0.5 and 1 µM), with 25 nM SIINFEKL peptide, with 100 nM RGYVYQGL peptide, with 1 µM TLM-eGFP or were left untreated. Forty-eight hours later, expression of CD69 (*a*) and 72 h later production of IFN-$\gamma$ by V$\alpha$2+ CD8+ T cells (*b*) were analyzed using flow cytometry. Mean fluorescent intensity of CD69 expression and percentage of IFN-$\gamma$-producing specific CD8+ T cells (*b*) were determined. One representative experiment of six independent experiments is shown (*with one exception: incubations with RGYVYQGL peptide and TLM-eGFP were tested only twice). (*c*) Splenocytes of OT-I mice were CFSE labeled and cultured with recombinant ovalbumin proteins (1 µM), with 250 nM SIINFEKL peptide or left untreated. At day 3, proliferation of specific CD8+ T cells was analyzed by flow cytometry. In the histograms, the percentage of proliferating V$\alpha$2+ CD8+ T cells (M2) is calculated. One representative experiment of three is shown.

protein (10 µg per mouse), OT-I T cells proliferated better after immunization with TLM-ova as compared to ova (51.5% versus 36.2%, respectively). This difference was less pronounced when higher protein concentrations were used (40–150 µg recombinant ova protein; data not shown).

Based on these findings we analyzed whether the immunization with TLM-ova also improved the generation of cytotoxic effector cells *in vivo*. We adoptively transferred the equivalent of $5\times10^6$ OT-I T cells into C57BL/6 mice and immunized the animals with 125 µg recombinant ova proteins (i.p.). Seven days later, cytotoxic activity of transferred OT-I T cells was analyzed by an *in vivo* cytotoxicity assay. No lysis of specific target cells could be detected in mice without adoptive transfer of OT-I T cells. After adoptive transfer of OT-I T cells but without immunization there was some background cytotoxicity (35.5% lysis of SIINFEKL-loaded target cells). However, immunization of mice with cell-permeable TLM-ova induced generation of specific cytotoxic activity (specific lysis 76.7%) that was well above the background and higher than that of animals immunized with ova (specific lysis 50.4%) (Fig. 4). These data demonstrate that *in vitro* as well as *in vivo*, the TLM enhances the immunogenicity of the protein antigen ovalbumin.

**TLM-ova vaccination improves protection against outgrowth of EG7-ova tumors in mice.** To address the question whether the enhanced immunogenicity of TLM-ova results in a better anti-tumor immune response after vaccination, C57BL/6 and OT-I mice were immunized s.c. with 100 µg of the respective recombinant ova protein plus CpG-ODN as adjuvant. Control groups remained untreated or were immunized with adjuvant only. Four-

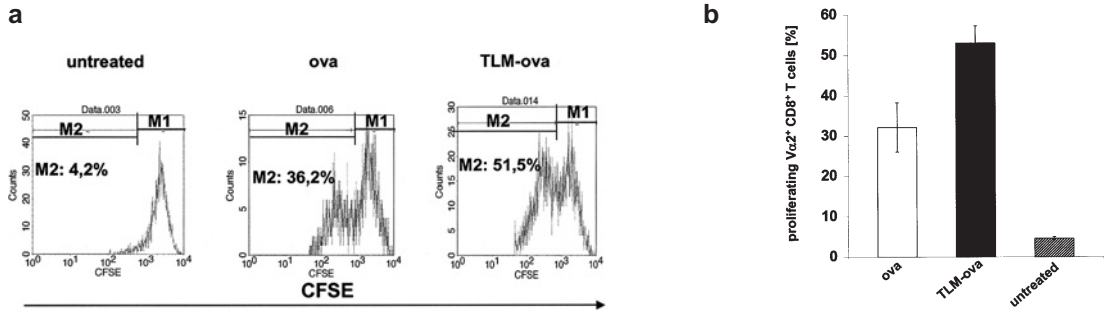
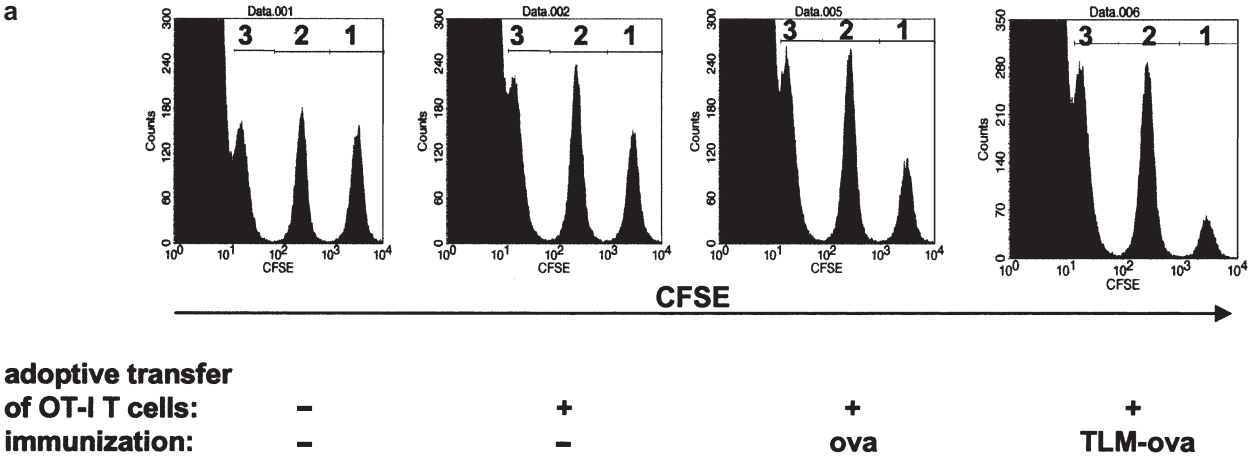



**Figure 3.** TLM-ova enhances proliferation of adoptively transferred OT-I T cells. (*a, b*) CFSE-labeled OT-I splenocytes were adoptively transferred in C57BL/6 mice (equivalent of $5\times10^6$ OT-I T cells per mouse). Eighteen hours after cell transfer, mice were immunized i.p. with 10 µg recombinant ovalbumin proteins without adjuvant. Control animals remained untreated. Fifty-two hours after immunization, proliferation of transferred CD8+ T cells in spleen was analyzed by flow cytometry. The percentage of proliferating V$\alpha$2+ CD8+ T cells (M2) was determined. The original data for one representative animal of each group are shown (*a*). One representative experiment of three is summarized in the bar diagram (n = 5 animals) (*b*).



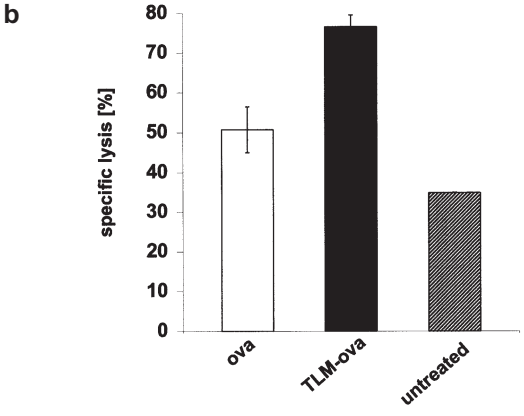




**Figure 4.** TLM-ova enhances cytotoxic activity of adoptively transferred CD8+ OT-I T cells. (*a, b*) OT-I splenocytes were adoptively transferred in C57BL/6 mice (equivalent of $5\times10^6$ OT-I T cells per mouse). Eighteen hours after cell transfer, mice were immunized with 125 µg recombinant ovalbumin proteins, i.p. without adjuvant. Control animals remained untreated. Seven days after immunization, cytotoxic activity of transferred OT-I T cells was examined in an *in vivo* cytotoxicity assay. The percentage of the three differentially labeled fluorescent target populations was analyzed by flow cytometry (1, target cells labeled with 5 µM CFSE/pulsed with 10 µM SIINFEKL; 2, target cells labeled with 0.5 µM CFSE/pulsed with 10 µM RGYVYQGL; 3, target cells labeled with 0.05 µM CFSE/not pulsed). The specific lysis was calculated. Histograms are shown for one representative animal of each group (*a*). One representative experiment of three is summarized in the bar diagram (n = 3 animals) (*b*).

teen days after immunization, mice were challenged s.c. with $5\times10^5$ ovalbumin-expressing EG7-ova cells and tumor outgrowth was determined for the following 60 days. Cumulative results from three (C57BL/6 mice) and two (OT-I mice) independent experiments, respectively, are shown in Table 1. Tumors grew progressively in almost all mice that were not immunized (14 of 15 mice in the C57BL/6 group and 8 of 8 mice in the OT-I group), as well as in mice treated with CpG-ODN alone (4 of 4 mice in the C57BL/6 group and 7 of 8 mice in the OT-I group). After immunization with recombinant ova proteins, the overall anti-tumor effects in C57BL/6 mice were moder-

ate. However, immunization of C57BL/6 mice with TLM-ova resulted in a delayed and a slightly reduced tumor outgrowth (Table 1). These differences were more pronounced in the TCR-transgenic OT-I mice, carrying a high ovalbumin-specific CD8+ T cell precursor frequency. All mice immunized with ova developed a tumor (0 of 8 animals tumor free). Nearly half of the animals remained tumor free after immunization with cell-permeable TLM-ova (4 of 9 mice tumor free). From these experiments, we can conclude that TLM-ova vaccination is superior to ova vaccination.

**Table 1.** TLM-ova vaccination enhances protection from outgrowth of EG7-ova tumors.

| Tumor-free animals after EG7-ova challenge (total animals per experimental group) | | | |
|---|---|---|---|
| Immunization | day 20 | day 40 | day 60 |
| C57BL/6 mice | | | |
| None | 1 (15) | 1 (15) | 1 (15) |
| CpG-ODN | 0 (4) | 0 (4) | 0 (4) |
| Ova | 4 (14) | 2 (14) | 2 (14) |
| TLM-ova | 7 (14) | 5 (14) | 4 (14) |
| OT-I mice | | | |
| None | 0 (8) | 0 (8) | 0 (8) |
| CpG-ODN | 1 (8) | 1 (8) | 1 (8) |
| Ova | 2 (8) | 1 (8) | 0 (8) |
| TLM-ova | 6 (9) | 4 (9) | 4 (9) |

Animals were immunized s.c. with 100 µg recombinant ovalbumin proteins plus CpG-ODN as adjuvant. Fourteen days after immunization, mice were challenged s.c. with $5\times10^5$ EG7-ova cells. Cumulative results of tumor growth are shown in the table (three independent experiments for C57BL/6 mice and two experiments for OT-I mice).

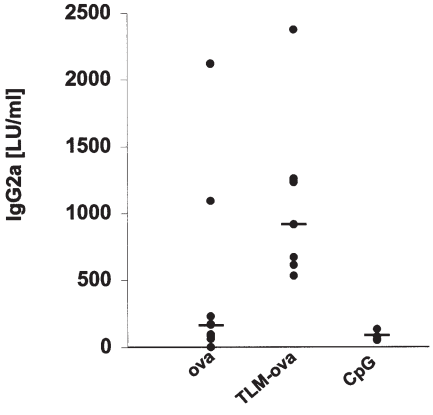



**Figure 5.** Vaccination with TLM-ova increases anti-ovalbumin IgG2a antibody titers. Blood of C57BL/6 mice was collected before and 14 days after immunization with recombinant ovalbumin proteins. Ovalbumin-specific IgG2a antibody titers in the sera were measured by ELISA. Before immunization, no ovalbumin-specific IgG2a antibody titers were detectable (data not shown). After immunization, antibody titers of two experiments (n = 6 animals in each experiment) are shown [the median of the IgG2a level in the untreated group was >3.5 LU/ml (not shown); in the CpG-ODN immunized group 80 LU/ml; in the ova-treated group 175 LU/ml and in the TLM-ova immunized group 919 LU/ml].

**Vaccination with TLM-ova increases anti-ovalbumin IgG2a antibody titers.** To analyze the effect of TLM-ova vaccination on the humoral response, the titer of anti-ovalbumin antibodies was determined. Serum of C57BL/6 mice was collected before and 14 days after immunization. Ovalbumin-specific IgG2a antibody titers in these sera were measured by ELISA. No ovalbumin-specific IgG2a antibodies titers could be detected in the blood of mice before immunization (data not shown). The titer of ovalbumin-specific IgG2a antibodies of mice immunized with TLM-ova showed elevated titers in the blood (median of IgG2a level: 919 LU/ml) as compared to the titer of animals immunized with ova (median of IgG2a level: 175 LU/ml) (Fig. 5). This indicated that not only CD8+ T cell-mediated responses are improved by TLM-ova vaccination but T helper cell activity and humoral immune responses (regarding IgG2a antibody titers) are improved as well.

## Discussion

Recent reports describe the potential of the HBV-derived translocation motif (TLM) for protein transfer [12–15]. In this study, we investigated whether this cell-permeable peptide can also be used to enhance immune responses to protein antigens when used for vaccination.

DCs are the central regulators of T-cell-mediated immune responses. Several vaccination strategies have been developed to transfer protein antigens into DCs *in vitro* [17–19]. Cell-permeable peptides have also been tested for antigen transfer into DCs. For example, Shibagagki and Udey [22, 23] recently demonstrated that the CPP of HIV-tat can efficiently translocate antigens into the cytoplasm of DCs. They showed in two independent tumor models that using DCs loaded with antigens containing HIV-tat CPP for vaccination enhanced CD8+ T-cell-mediated immune responses. Since generation of DCs for vaccination and *in vitro* loading is cumbersome, we analyzed whether direct application of cell-permeable proteins results in an improved loading of APCs *in vivo* and thereby enhances T cell immunity compared to application of the corresponding non-cell-permeable protein.

The CPP TLM is well suited to analyze how a CPP affects immunogenicity if used for vaccination, for the following reasons. (i) There has been no size limitation observed for cargos fused to the TLM. (ii) TLM-mediated transfer does not show any preference for subcellular compartments and does not affect the functionality of its cargo. (iii) The TLM displays no cell toxicity, no immunogenicity and does not interfere with intracellular signaling [12]. (iv) In contrast to tat-mediated transfer, TLM-dependent transfer does not involve endocytotic steps [9–

11]. Therefore, TLM-dependent transfer displays no restrictions on transfer in terms e.g. of differentiation state or membrane composition of cells.

As a model antigen we decided to use the ovalbumin protein fused to the TLM to test the effect of CPP for vaccination because the cellular immune responses to ovalbumin can be easily evaluated in established models such as OT-I mice, and humoral immune responses can be evaluated by ELISA. There are two reports describing the application of the antennapedia-derived CPP fused to a peptide for vaccination in the ovalbumin model system [21, 27]. Both studies found that cellular immune responses are improved. However, it was unclear whether CPPs are also suitable to improve the immune response to complete proteins. Using proteins in contrast to peptides offers the advantage that specific epitopes do not have to be characterized and, furthermore, presentation does not have to be restricted to certain haplotypes. The results of this study show that fusion of the TLM to ovalbumin improved CD8+ T-cell-mediated immune responses *in vitro* and *in vivo* as compared to ova. Although the TLM shows no APC selectivity for the translocation process, our results suggest that the TLM is a suitable transfer system for direct or indirect antigen transport into APCs *in vitro* and *in vivo*, because immune responses can be triggered by TLM-ova. TLM-protein vaccination-induced anti-tumor effects were moderate compared to other vaccination strategies. Using cytokine-gene-modified cells for vaccination [31] or antigen-pulsed DCs [23, 25], the majority of the mice can be protected from outgrowth of tumors. Nevertheless, using TLM-ova we observed a delayed tumor outgrowth in C57BL/6 mice after immunization compared to non-cell-permeable proteins. These effects were more pronounced in OT-I mice that carry an ova-specific TCR. Additionally, the humoral immune response, at least for IgG2a antibody production, was improved after immunization with cell-permeable proteins.

We do not know the mechanisms whereby the TLM enhances immunogenicity of protein antigens. Possible explanations are e.g. an increased uptake of TLM fusion proteins in APCs, altered effects on the antigen release kinetics or an increased immunogenicity of the TLM fusion antigens themselves. It will be important to elucidate the functional mechanisms of TLM fusion antigens. Then the observed effects may be improved by rational design of TLM fusion antigens, inserting the TLM in different regions of the protein (N/C terminus) or altered immunization protocols. In summary, our study demonstrates that TLM fusion to the model antigen ovalbumin enhances CD8+ T cell activation and function *in vitro* as well as *in vivo* and improves vaccination efficacy. Generation of cell-permeable TLM-protein variants may therefore represent a promising tool for the improvement of the cellular and humoral immune responses in protein-based vaccination strategies.

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