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Efficient cross-presentation of soluble exogenous antigens introduced into dendritic cells using a weak-based amphiphilic peptide

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ABSTRACT

To develop a novel dendritic cell (DC)-based vaccine for inducing antigen-specific $CD8^+$ T cell responses by cross-presentation, we tested a novel antigen delivery system that introduces soluble antigens into the cytosol of cells by an endocytosis-mediated mechanism which avoids damaging the plasma membrane ("Endo-Porter"™). Proteins released from endosomes into the cytoplasm are degraded by the proteasome, and fragmented antigenic peptides are presented to the classical cytosolic MHC class I pathway. DCs pulsed with OVA protein in the presence of Endo-Porter efficiently stimulate OVA peptide-specific $CD8^+$ T (OT-I) cells. Although this agent diverts some of the endocytosed antigens away from the classical MHC class II-restricted presentation pathway to the class I pathway, the activation of $CD4^+$ T cells was found not to be hampered by Endo-Porter-mediated antigen delivery. On the contrary, it was rather augmented, probably due to the increased uptake of antigen. Because specific $CD4^+$ T cell help is required to license DCs for cross-priming, Endo-Porter-mediated antigen delivery is a promising approach for developing more efficient cancer vaccines targeting both $CD4^+$ and $CD8^+$ T cells.

Introduction

A major goal of tumor immunotherapy is the generation of functional cytotoxic T lymphocytes (CTLs) that efficiently kill tumor cells. Dendritic cell (DC)-based cancer vaccines are being extensively employed in the development of approaches to generate tumor-specific CTLs [1]. However, DCs pulsed with soluble antigens preferentially stimulate $CD4^+$ rather than $CD8^+$ T cells. Therefore, we conducted the present study to develop a DC-based vaccine that can induce antigen-specific $CD8^+$ T cell responses by facilitating antigen cross-presentation.

In general, peptides derived from cytosolic proteins synthesized within DCs and cleaved by proteasomes are loaded onto MHC class I molecules to be presented to $CD8^+$ T cells, while exogenous proteins taken up by DCs are proteolytically fragmented in acidic endosomes. The resulting peptides are then loaded onto MHC class II molecules to be presented to $CD4^+$ T cells [2]. However, a fraction of the exogenous protein internalized by DC is also processed and displayed as peptide-MHC class I complexes [3]. This process is referred to as "cross-presentation" [4]. Exogenous proteins taken up by DCs are transferred from endocytic compartments to the cytosol [5–7]. Cytosolic antigens are degraded by the proteasome and the resulting peptides are translocated into the lumen of the endoplasmic reticulum (ER) or phagosomes by the transporter associated with antigen processing (TAP). They are then loaded onto MHC class I molecules and presented at the cell surface [4,8–10]. Cross-presentation probably plays a crucial role in initiating $CD8^+$ T cell responses to tumors and virus-infected cells. Therefore, the development of strategies aimed at increasing the efficacy of priming $CD8^+$ T cell responses is essential for improving DC-based vaccination. To this end, here we develop a technique for external soluble antigen delivery into the endosomal compartment and/or cytoplasm of DCs using Endo-Porter™ (GENE TOOLS, LLC., Philomath, OR).

Endo-Porter is a weak-base amphiphilic peptide that was designed to deliver non-ionic substances such as peptides and proteins into the cytoplasm of cells by an endocytosis-mediated process [11]. Endo-Porter binds to the plasma membrane by its lipophilic face and is rapidly endocytosed, along with any substances present in the medium. The weak-base face of Endo-Porter exists in a non-ionic state in the extracellular medium (about pH

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7.2–7.5), while in the endosome it is converted to its poly-cationic form on the weak-base face upon acidification (about pH 5.0–6.0). This results in the permeabilization of the endosomal membrane and release of the co-endocytosed proteins into the cytosol of the cell. Taking advantage of these characteristics that can shuttle exogenous protein into the cytosol (endogenous pathways), we used Endo-Porter as a vehicle for delivering antigens into DCs.

We tested Endo-Porter as a delivery vehicle for MHC class I-restricted cross-presentation. We used both bone marrow-derived DC and splenic $CD8^-$ DC as antigen presenting cell (APC) in these experiments. We demonstrate that Endo-Porter enhances cross-presentation, which is inhibited by dimethyl amiloride (DMA), MG132, and brefeldin A, but is resistant to chloroquine or primaquine. The cross-presentation was abrogated in TAP1 deficient ($TAP1^{-/-}$) DCs. We conclude that Endo-Porter acts as an excellent delivery vehicle for proteasome-TAP dependent cross-presentation of exogenous antigens.

Materials and methods

Mice. C57BL/6 mice at the age of 6–8 wk were obtained from Japan SLC (Sizuoka, Japan) and used for generation of DCs. $TAP1^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). TCR-transgenic C57BL/6 OT-I mice (C57Bl/6-RAG2tm1Fwa-TgN) and C57BL/6 OT-II (C57Bl/6-RAG2tm1Alt-TgN) mice were from Taconic (Hudson, NY) [12–14]. All animal procedures were conducted in accordance with the institutional guidelines.

Flow cytometric analysis. The following monoclonal antibodies were used for flow cytometry: FITC-conjugated anti-CD69, phycoerythrin-conjugated anti-CD4, anti-CD8, allophycocyanin-conjugated anti-CD8 (Biolegend, San Diego, CA). Cells were stained with antibodies and analyzed using a Cytomics FC 500 (Beckman Coulter, CA). The data were processed using CXP Analysis 2.0 software (Beckman Coulter).

DC generation. Bone marrow-derived DCs were prepared as described by Lutz et al. [15]. Briefly, bone marrow cells obtained from tibias and femurs of C57BL/6 or $TAP1^{-/-}$ mice were cultured in RPMI 1640 medium supplemented with 10% FCS, 12.5 mM HEPES, 5×10^{-5} M 2-mercaptoethanol, 1×10^{-5} M sodium pyruvate, 1% nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin and 20 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ) for 8 days. On days 3 and 6, half of the medium was replaced with fresh medium containing GM-CSF.

Assay for MHC class I and/or II antigen presentation. DCs (2×10^5) were incubated with the indicated antigens at 37 °C for the indicated times in 5 ml polystyrene round-bottom tubes (Falcon 2058). OVA protein was purchased from Sigma Aldrich Japan (Tokyo, Japan). The OT-I ($OVA_{257–264}$, SIINFEKL) and OT-II ($OVA_{323–339}$, ISQAVHAAHAEINEAGR) peptides were purchased from Invitrogen Japan (Tokyo, Japan) at purities of >90%, with a free amino terminal and a carboxy-terminal. Antigen-pulsed DCs were washed with HBSS and returned to culture in medium containing 10 ng/ml GM-CSF, 10 ng/ml IL-4 (PeproTech) and 1 μg/ml lipopolysaccharide (LPS; Sigma) for 4 h to generate mature DCs. DCs were then incubated with spleen cells from OT-I/$RAG^{-/-}$ or OT-II/$RAG^{-/-}$ mice for 16 h. Cells were harvested and stained for CD4, CD8, and CD69 and analyzed by flow cytometry. DCs and T cells were also co-cultured for 3 days and IL-2 production in the culture supernatant was quantified using murine IL-2 ELISA kits (Biolegend).

To examine the effects of Endo-Porter on intracellular antigen processing, dimethyl amiloride (DMA) (Sigma), MG132 (Cosmo Bio, Tokyo), Z-FA-FMK (Cosmo Bio), brefeldin A (Sigma), chloroquine (Sigma), or primaquine (Wako Pure Chemical Industries, Osaka, Japan) were added to DCs for 30 min prior to and throughout the 2 h antigen pulse. Following treatment with the inhibitors, the DCs were fixed in 0.008% glutaraldehyde (Wako), and then washed ×4 with cold RPMI 1640. The DCs were co-cultured overnight with OT-I cells after which CD69 expression by the latter was evaluated.

Isolation of splenic $CD8^-$ DCs. First, DCs were enriched by depletion of T cells, NK cells, and B cells with a cocktail of biotin-conjugated antibodies against CD90 (Thy1.2), CD49b (DX5), and CD45R (B220) as well as anti-biotin microbeads (Miltenyi Biotec, Auburn, CA). In addition, CD8α microbeads were added to deplete $CD8^+$ cells. Subsequently, $CD11c^+$ DCs ($CD8^-$) were positively selected by CD11c microbeads.

Statistical analysis. Statistical analyses were performed with JMP software, version 8.0 (SAS Institute Inc., Cary, NC, USA). Results are shown as mean ± SD. Comparison of results was carried out using the two-tailed unpaired *t*-test. $p < 0.05$ was considered statistically significant.

Results

Endo-Porter-mediated antigen delivery into the DC cytosol enhances cross-presentation

To develop a novel vaccine using DCs pulsed with soluble antigens, we tested whether delivering the antigen directly into the cytosol would enhance cross-presentation. For this, we used Endo-Porter, an agent that can shuttle antigens from outside the cell into endosomes, and from there into the cytosol [11]. First, imDCs were co-cultured with OVA (10 μg/ml) in the presence or absence of Endo-Porter for 6 h to allow them to capture, process and prepare to present antigens. After LPS stimulation, DCs were co-cultured overnight with OVA peptide-specific $CD8^+$ or $CD4^+$ TCR transgenic T cells (OT-I or OT-II, respectively). Antigen presentation via the MHC class II pathway was detected by the activation of OT-II cells, while cross-presentation by MHC class I molecules resulted in the rapid activation of OT-I cells. Activation of both was monitored by their expression of the early activation marker CD69 and the production of IL-2 (Fig. 1). CD69 was not upregulated on either TCR-transgenic OT-I or OT-II cells following coculture with unpulsed DCs without OVA, whereas OT-I or OT-II peptide-pulsed DCs efficiently induced CD69 on the respective T cells (Fig. 1A and B). These results confirm that antigen-specific activation of OT-I and OT-II cells can be effectively monitored by measuring their CD69 expression.

The frequency of $CD69^+$ OT-I cells was only 5.0 ± 0.8% when they were incubated with DC alone (Fig. 1A). In contrast, because OVA is constitutively cross-presented to some extent, DCs pulsed with the protein (10 μg/ml) did induce CD69 expression on 32.8 ± 16.4% of OT-I cells. CD69 expression on these cells was markedly increased by coculture with DC that had been pulsed with OVA in the presence of 1 μM Endo-Porter (84.8 ± 10.2%). This was similar to the level induced by DCs that had been pulsed with 1 μg/ml of the OT-I peptide itself (96.6 ± 5.6%). That this was a specific effect is documented by the finding that DCs treated with Endo-Porter alone failed to induce high level CD69 expression on OT-I cells (Fig. 1C). These results suggest that Endo-Porter-mediated antigen delivery markedly enhances cross-presentation of soluble antigen by DCs.

Next, we measured the frequency of $CD69^+$ OT-II cells after stimulation with DC alone, DC pulsed with OVA (10 μg/ml) in the presence or absence of Endo-Porter (1 μM), or OT-II peptide (1 μg/ml). These values were 3.5 ± 1.9%, 51.5 ± 19.5%, 64.3 ± 19.4% and 76.9 ± 17.0%, respectively, again indicating that DCs efficiently captured and processed OVA. As expected, OVA was efficiently processed and presented by MHC class II molecules to OT-II cells.