SHORT REPORT

Targeting Human Dendritic Cells with Lewis X Modified Liposomes

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L.K. designed the research, designed and performed experiments, analyzed data and wrote the manuscript

Y.Z. selected the most suitable Lewis X bearing glycolipid and synthesized and characterized the compound, and contributed to writing the manuscript

Abstract

In this short report, we demonstrate that liposomes bearing the Lewis X trisaccharide on the surface ("Awesosomes") efficiently target human dendritic cells. We chose a glycolipid with Lewis X trisaccharide headgroup to facilitate the targeted liposome uptake via the DC-SIGN internalization pathway. While no uptake of Awesosomes was detected with wild-type human HEK293 cells, HEK293 cells transfected with human DC-SIGN internalized Awesosomes extensively. In samples of human blood-derived leukocytes, the extent of uptake of Awesosomes correlated with the expression of DC-SIGN, which is a dendritic cells marker. There was a marked difference in the uptake of Awesosomes and plain liposomes by DC-SIGN expressing dendritic cells. There was no difference in uptake of Awesosomes and plain liposomes by wt HEK293 cells or macrophages. These results indicate that Lewis X trisaccharide can "sweet-talk" dendritic cells into internalizing a delivery vehicle, and that Awesosomes are promising as "magic bullets" for specific delivery of drugs, antigens, or immunostimulatory molecules to human dendritic cells without influencing other cell types.

Introduction

Dendritic cells (DCs) are the most powerful antigen presenting cells. They are extremely potent in priming naïve T-cells (and have been therefore designated the "nature's adjuvants" [Steinman, 2007], and in inducing cytotoxic immune responses (CTL). Cytotoxic response is required for an efficient protection against a number of pathogens that evade immune response, and also against cancer. Importantly, DCs are capable of cross-presentation, the induction of a cytotoxic cell response against exogenously acquired antigen [Bevan, 1976]. This mode of CTLs induction against an exogenous antigen has been described only relatively recently and is extremely important for developing vaccines against certain pathogens including malaria, HIV, and TB [Winau, 2006], but also against tumor cells.

In addition to antigen targeting to DCs, which has already been described [Bonifaz, 2004; Sancho, 2009] and shown to increase vaccine performance, we believe that also targeting of immunostimulatory molecules and other adjuvants may be beneficial. Targeted delivery of immunostimulants should decrease the likelihood of mounting a generalized inflammatory response; current adjuvants elicit a generalized inflammatory response, often leading to a number of side effects like swelling.

Thus, our aim was to find a way to target a universal delivery vehicle suitable for delivery of both antigens and adjuvants to DCs. Liposomes are an ideal vehicle because they provide the interior aqueous environment for encapsulation of hydrophilic molecules (e.g. protein and peptide antigens, or soluble immunostimulatory molecules like CpG or poly I:C), and the lipid membrane, which can harbor hydrophobic molecules (acyl-modified peptides, TLR4 agonists derived from lipid A, etc.).

We chose to target DC-SIGN receptor, a C-type lectin for which the ligand profile has been thoroughly characterized and high-affinity glycan ligands have been identified [Guo, 2004]. Lewis X trisaccharide has been identified as one of the best DC-SIGN ligands. Esnault et al. described the synthesis of highly hydrophic Lewis X glycolipids [Esnault, 2001] which bind DC-SIGN. We show that POPC liposomes with 0.5mol% of this Lewis X compound are efficiently taken up by human blood derived dendritic cells.

Materials and Methods

Preparation of plain liposomes and Awesosomes

Lipids were mixed in chloroform and dried under a stream of nitrogen. The resulting film was hydrated with 20mM

HEPES/130mM NaCl, pH 7.25, vortexed, sonicated, and allowed to go through 1 to 5 freeze-thaw cycles before extrusion through 100nm polycarbonate membrane at a temperature equal or higher than that of the Tm of the lipids.

Transfection of HEK293 cells with DC-SIGN

HEK293 cells were seeded at a density of ~25000 cells / cm^2. Next day, they were transfected using the FuGENE (Roche) transfection reagent as follows: 1.5µg pcDNA3.1 containing the human DC-SIGN (a kind gift of Benhur Lee, LA) was mixed with 4.5µl FuGENE in 100ul serum-free MEM media, and allowed to complex for 10-30'. The mixture was then added to the cells in complete media (0.1µg DNA was enough for 1cm^2 cells). The transfected cells were then incubated O/N to allow for the expression of DC-SIGN.

Cell imaging and immunofluorescence

In order to visualize the uptake of liposomes, 0.05% of DiO-C_{18} or DiI-C_{18} (Invitrogen) was included in the lipid mix. For viewing, the cells were washed extensively with PBS/0.2% BSA/0.5% gelatin/2mM EDTA, fixed with 4% PFA (DAPI was included when required), and when appropriate, blocked with PBS/gelatin and stained with FITC-conjugated anti-DC-SIGN antibody (R&D FAB161F, used at a recommended dilution). The cells were then washed again and finally mounted in mowiol for viewing.

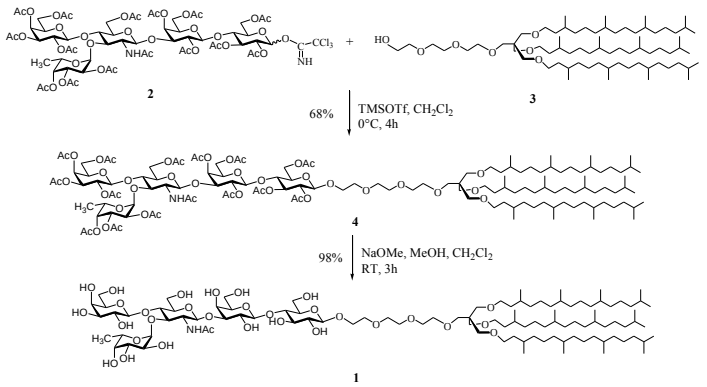
Liposome internalization assays

Cells were grown in 96 well plates to confluency. Liposomes were added at a concentration of 0.3mM lipid, mixed, and incubated as indicated. When the incubation period was over, the cells were chilled quickly on ice, washed extensively with PBS/gelatin/EDTA, and the fluorescence was measured in PBS.

New synthetic method of Lewis X neolipid

The Lewis^x neolipid **1** used in this study was originally synthesized by our group for study of its monolayer behaviour [Esnault, 2001]. A more efficient synthetic route for this compound has been developed recently in our group which was depicted in Scheme 1.

The known pentasaccharide trichloroacetimidate **2**, readily obtained by our previous report [Gourier, 2005], was condensed with the known alcohol **3** [Esnault, 2001]. The reaction was promoted by trimethylsilyl triflate (TMSOTf) in dichloromethane in the presence of molecular sieve (4Å) at 0°C for 4h to give the protected Lewis^x neolipid **4** in 68% yield. De-O-acetylation of compound **4** by sodium methoxide in methanol and dichloromethane at room temperature for 3h provided the target product **1** in 98% yield. Both compounds **1** and **4** were fully characterized by ^{1}H NMR, ^{13}C NMR and Mass Spectroscopy analyses.



Scheme 1

Results and Discussion

DCs express multiple surface receptors that are endocytosed upon ligand binding – among others the ubiquitous mannose receptor and other C-type lectin receptors including some DC-specific receptors like DC-SIGN. Many of those receptors can be used for the desired targeted uptake, and some of them will favor cross-presentation. It has been shown by conjugating an antigen to anti-DEC-205 antibody that it is indeed possible to increase the efficiency of vaccination for T cell immunity by targeting the antigen to DCs [Bonifaz, 2004]. However, our objective was **to achieve a "protein-free" targeting**, and to develop a system that is flexible and easily customizable. Using a saccharide structure to target a lectin receptor seems to be a reasonable choice. In the past, branched oligo-mannose and fucose structures, which are common in pathogens, were used both on liposomes and emulsions [White, 2006; Yeeprae, 2005; Copland, 2003]. The use of such non-specific structures was effective in enhancing particle uptake in general because these glycans are recognized by a number of surface lectin receptors, however, these molecules do not provide the desired efficient targeting effect. It is also important to keep in mind that in order to achieve the desired CTL immunity, the target receptor has to direct the cargo into the MHC I pathway, which is not the case with the mannose receptor [Sallusto, 1995].

We felt that the time is now ripe to revisit the idea of modification of liposomal surface with oligosaccharide structures while taking an advantage of the recent advances in identification and characterization of lectin-type receptors on the surface of DCs. Our approach is

novel in that the oligosaccharide structures that we employ are not generic structures targeting a broad range of receptors, but are rationally designed to target a known, specific receptor (DC-SIGN) on a known type of cells (DCs). According to the excellent study by Guo et al. [Guo, 2004], Lewis a and Lewis X trisaccharides are efficiently recognized by DC-SIGN while escaping binding to the DC-SIGN homologue DC-SIGNR, which is expressed on endothelial cells. We chose the glycolipid first introduced by Esnault et al. [Esnault, 2001] as the targeting signal. This compound has a pentasaccharide headgroup composed of the Lewis X trisaccharide determinant connected via a lactose group to the lipid backbone (**1**, Scheme 1). Similar approach was reported to target model protein antigen, ovalbumin, to DC-SIGN by conjugating Lewis X antigen [Wang, 2006; Singh, 2009]. The results obtained with Lewis X-targeted antigen are highly encouraging.

In general, the amount of the vaccine components that the cells internalize will correlate with their concentration at the site of injection and with their endocytic/pinocytic/phagocytic activity. Thus, in order to limit the uptake of a vaccine component to DCs, efficient targeting signal is needed. Ideally, the vehicle should not be readily internalized by any other cells than DCs.

In order to gain maximum benefit from the targeting effect of the Lewis glycolipid (Lx), we were looking for liposomes that are internalized poorly, especially by macrophages. Hence we avoided positively charged lipids because the liposomes with positive charge are readily taken up by all cells and thus the targeting capacity of the Lewis glycolipid would be masked. We tested 10 different lipid compositions in RAW 264.7 macrophages and mouse bmDCs. The compositions were based on our experience and available literature, and the uptake was followed by measuring fluorescence (Figure 1).

In order to ensure that the measured fluorescence corresponded to endosomal uptake of the liposomes, we confirmed the results by microscopy (Supplemental figure 1). We chose simple POPC liposomes, because the kinetics of uptake were similar to transferrin, the total amount internalized was relatively low and very evenly distributed among the cells, and there were no aggregates. In addition, POPC liposomes are stable and non-toxic. Also binary mixtures of POPC and cholesterol gave good results (Figure 1) but we used POPC for simplicity. However, it is clear that the uptake can still be fine-tuned by changing the composition. In addition, also the size of the liposomes is an important factor – because the idea was to target DCs via receptor mediated endocytosis, we used liposomes of ~100nm diameter (large unilamellar vesicles, LUVs). Considerably larger liposomes could be preferentially taken up by phagocytosis.

Next we tested the targeting effect of POPC liposomes with 5 mol% Lx. We used wt HEK293 cells and HEK293 cells transfected with human DC-SIGN plasmid. The results were remarkably unambiguous. Virtually no uptake of plain POPC liposomes was observed with either wt or DC-SIGN transfected HEK293 cells, and the same result was obtained with POPC-Lx liposomes ("Awesosomes") in wt HEK293 cells. However, copious amounts of Awesosomes were internalized by DC-SIGN expressing HEK293 cells (Figure 2).

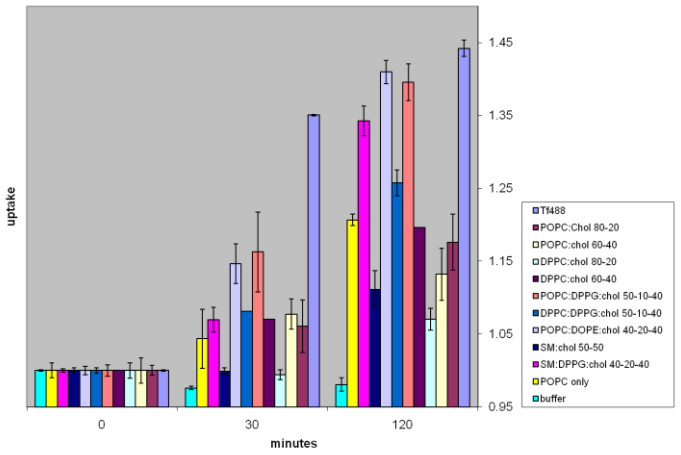


Figure 1 Uptake of liposomes of different lipid compositions by RAW 264.7 macrophages. All liposomes were approximately 120nm in diameter. As a reference, we used Alexa488-conjugated transferrin (Tf488). The uptake was quantified by measuring fluorescence.

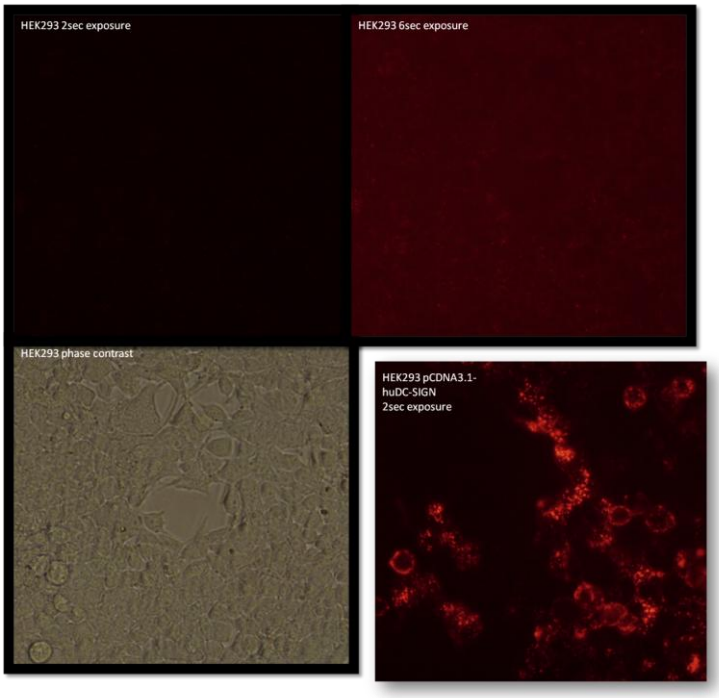


Figure 2 Uptake Awesosomes by wt and huDC-SIGN expressing HEK293 cells The 3 panels on top left, top right, and bottom right show one and the same view-field of the wt HEK293 cells that were exposed to Awesosomes. The bottom right panel shows HEK293 cells transfected with human DC-SIGN that were exposed to Awesosomes under identical conditions (incubation time ~30', 37°C).

Finally we tested the uptake by human blood derived DCs. DCs were derived by established methods either by selecting $CD14^+$ monocytes using magnetic αCD14 beads and subsequently differentiating into DCs, or by plate adherence selection followed by differentiation (IL-4 + GM-CSF). We obtained similar results with DCs prepared either way. The results were virtually identical to the results obtained with HEK293 cells: no uptake of Awesosomes was observed with cells that did not stain for DC-SIGN, while uptake of Awesosomes by DC-SIGN positive cells (ie DCs) was robust (Figure 4). This was established by microscopy, and then further validated by flow cytometry analysis which quantitatively confirmed the correlation of Awesosome internalization with surface expression of DC-SIGN (Figure 4, bottom right).

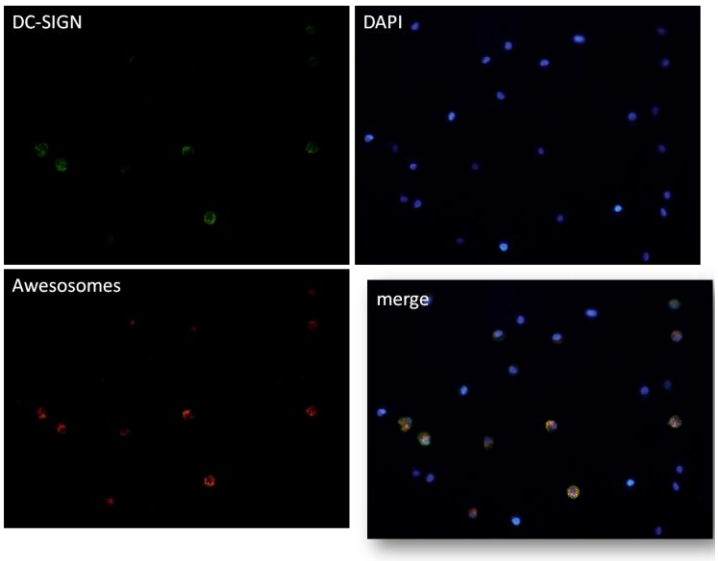


Figure 3 Only cells expressing DC-SIGN internalize Awesosomes A sample of human blood-derived DCs (80-90%) was given Awesosomes and subsequently fixed and stained with DAPI (stains all cells, blue) and anti-DC-SIGN antibody (stains DCs, green). It is clear that the expression of DC-SIGN correlates with the internalization of Awesosomes

In addition, we investigated how much of the Lx-glycolipid needs to be included to provide a sufficient targeting effect. We tested 0.05%, 0.1%, 0.5%, 1% and 5% (fractions expressed as molar %). No targeting effect was observed with 0.05%, and the difference between uptake of plain liposomes and liposomes with 0.1% was not convincing. However, using 1% appeared to be equally good as 5%, and the difference between 0.5% and 1% was only marginal as judged by microscopy (data not shown). Thus the optimal targeting concentration lies between 0.1 and 0.5%, which corresponds to approximately 100 to 500 molecules in the outer leaflet.

Taken together, we have shown that Lx-POPC Awesosomes efficiently target human monocyte-derived dendritic cells. The same approach could be possibly used to target endocytic lectin-type receptors other than DC-SIGN. We are sure that this will be a fruitful area of research in the future.

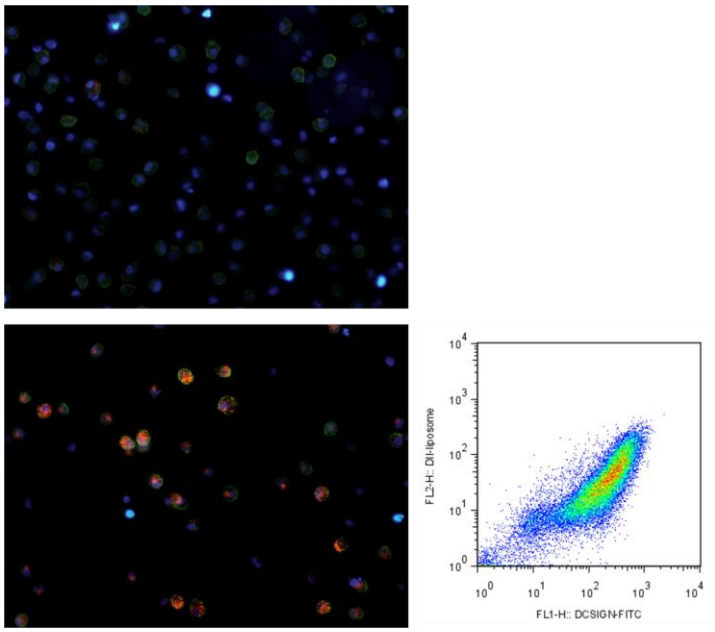


Figure 4 Uptake of plain liposomes and Awesosomes by human blood-derived DCs Human blood-derived DCs were incubated with plain liposomes (top) or Awesosomes (bottom) for ~30', washed, fixed, and stained with DAPI (blue, stains all cells) and anti-DC-SIGN (green, stains dendritic cells). The panel on the right the same sample analyzed by flow-cytometry; for compensation, single stained and unstained controls were used.

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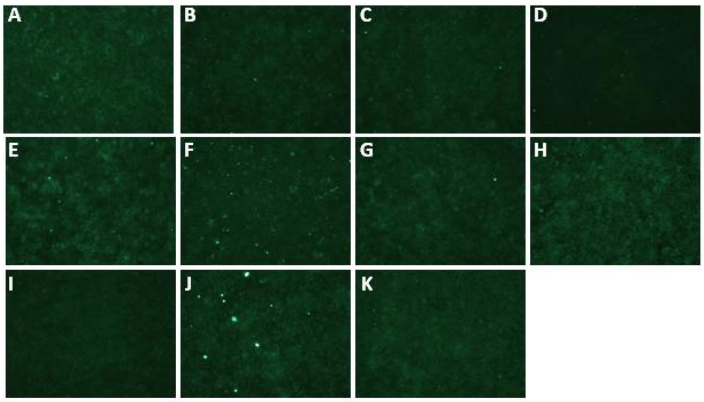
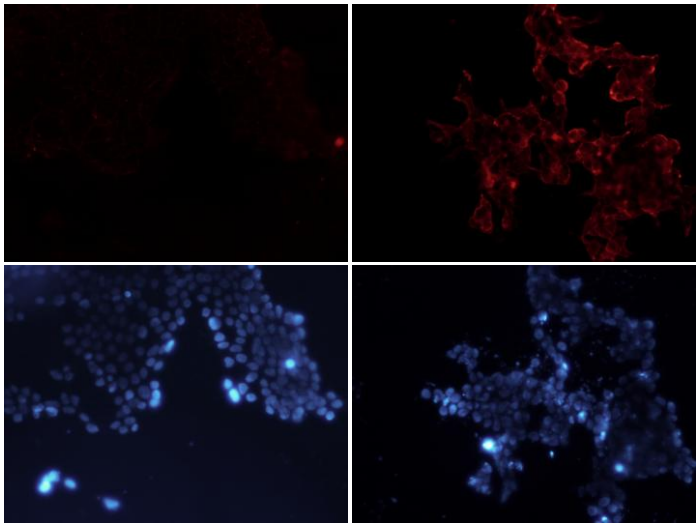
Supplemental figures



Supplemental figure 1 Uptake of liposomes of different lipid compositions by RAW 264.7 macrophages: Images A (transferrin) – K (POPC) show the wells with cells from Figure 1 at the 30' timepoint.



Supplemental figure 2 Binding of Awesosomes to wt and huDC-SIGN transfected cells: Left column – binding of Awesosomes to wt HEK293 cells; right column – binding of Awesosomes to huDC-SIGN transfected HEK293 cells (top – liposome chanel (DiI), bottom – DAPI)