Lewis X oligosaccharides targeting to DC-SIGN enhanced antigen-specific immune response

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Summary

Dendritic cell-specific intercellular-adhesion-molecule-grabbing non-integrin (DC-SIGN) is a potential target receptor for vaccination purposes. In the present study, we employed Lewis X (Le^x) oligosaccharides, which mimic natural ligands, to target ovalbumin (OVA) to human dendritic cells (DCs) via DC-SIGN, to investigate the effect of this DC-SIGN-targeting strategy on the OVA-specific immune response. We demonstrated that Le^x oligosaccharides could enhance the OVA-specific immune response as determined by enzyme-linked immunospot assay (ELISPOT), intracellular interferon-γ staining and ^{51}Cr-release assay. An almost 300-fold lower dose of Le^x-OVA induced balanced interferon-γ-secreting cells compared to OVA alone. Furthermore, secretion of interleukin-10, a reported mediator of immune suppression related to DC-SIGN, was not increased by Le^x-OVA, either alone or together with sCD40L-stimulated groups. A blocking antibody against DC-SIGN (12507) reduced the numbers of interferon-γ-secreting cells during Le^x-OVA stimulation, yet it did not prevent Le^x oligosaccharides from promoting the secretion of interleukin-10 that was induced by ultra-pure lipopolysaccharide. These results suggested that the strategy of DC-SIGN targeting mediated by Le^x oligosaccharides could promote a T-cell response. This DC-targeting may imply a novel vaccination strategy.

Keywords: dendritic cells; DC-SIGN; Lewis X; T cells; vaccination

Introduction

Targeting vaccine to dendritic cells (DCs) is an important and convenient strategy to enhance vaccine immunogenicity. The selection of an appropriate receptor is a principal factor for the successful vaccine targeting of DCs.[1,2]

Dendritic cell-specific intercellular-adhesion-molecule-grabbing nonintegrin (DC-SIGN), a C-type lectin-like receptor, is mostly expressed on immature DCs[3,4] and acts as an antigen receptor.[5,6] So far, studies indicate that DC-SIGN could efficiently capture a variety of pathogens, such as human immunodeficiency virus and cytomegalovirus virions at a low concentration in the mucosal tissues.[3,7,8] DCs can then present these antigens in major histocompatibility complex class II-restricted[4] or class I-restricted[5] fashions. However, following interactions between DC-SIGN and natural ligands, some immunomodulatory signals will be delivered to promote secretion of interleukin-10 (IL-10), inhibit maturation of DCs[9] and shift toward a T helper type 2 (Th2) immune response.[10] Although several features, including restricted expression on DCs and acting as an antigen receptor, make DC-SIGN an appropriate receptor for vaccination purposes,[11] a proper strategy utilizing DC-SIGN still needed to be developed.[9]

Lewis X (Le^x) oligosaccharide, a natural ligand of DC-SIGN, has higher affinity and specificity compared to the other oligosaccharide structures.[12–14] Le^x oligosaccharide is also an important component on the surface glycans of

Abbreviations: DC-SIGN, dendritic cell-specific intercellular-adhesion-molecule-grabbing non-integrin; ELISPOT, enzyme-linked immunospot assay; GM-CSF, granulocyte–macrophage colony-stimulating factor; HLA, human leucocyte antigen; IFN, interferon; IL, interleukin; K562-ED, K562 cell lines expressing DC-SIGN-EGFP protein; Le^x, Lewis X; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells.

soluble eggs antigen from *Schistosoma mansoni*, and its repetitive array has been reported to contribute to the induction of the production of Th2-associated antibodies and cytokines in BALB/c mice.[15–17] Lipopolysaccharide (LPS) containing Le^x sugar from *Helicobacter pylori* is found to be able to inhibit Th1 responses *in vitro* via DC-SIGN.[10] However, interactions between Mac-I on the neutrophils and DC-SIGN on the DCs mediated by Le^x sugar does not result in Th2-type polarization of effector T cells.[18] It is suggested that the Le^x oligosaccharides contained in a different expression system possibly produce different effects on DCs, and that Th2 biasing is not the only result of Le^x sugar treatment.

In the present study, we used a biotin–streptavidin (SA) system to conjugate Le^x oligosaccharides to ovalbumin (OVA) antigen, and then investigated the effect of this targeting strategy on OVA-specific T-cell responses *in vitro*. Our results indicated that the Le^x-OVA antigen could enhance antigen-specific $CD8^+$ T-cell immune responses by almost 300-fold compared with OVA alone. Moreover, secretion of IL-10 was not increased in the supernatant of effector DCs that were stimulated were with Le^x-OVA alone or in combination with CD40 ligand (CD40L) instead of ultra-pure LPS.

Materials and methods

Preparation of Le^x-OVA

According to the manufacturer's directions, SA (MERCK-Calbiochem, Darmstadt, Germany) was pretreated with 2-iminothiolane/HCl (Pierce, Rockford, IL) for 1 hr at room temperature, then allowed to react with the maleimide-activated OVA (Pierce) for 2 hr at room temperature to form the SA-OVA conjugates. The SA-OVA conjugates were purified by affinity chromatography with Sepharose 4B (Amersham Biosciences, Uppsala, Sweden) conjugated with anti-OVA polyclonal antibody and ultrafiltration (50 000 MW, Millipore, Bedford, MA). A Bradford micro-assay was used to quantify the SA-OVA conjugates.

The conjugates were confirmed by Western blot. In brief, conjugates were separated in 10–15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gel under non-dithiothreitol conditions and stained with Coomassie blue. The bands were transferred electrophoretically to polyvinylidene difluoride membranes and incubated with anti-OVA (Rockland, Gilbertsville, PA) and anti-SA (Vector, Burlingame, CA) antibodies. The results were visualized using chemiluminescence (Pierce).

The OVA protein was coated onto enzyme-linked immunosorbent assay (ELISA) plates to generate a standard curve for the quantification of OVA contained in the SA-OVA conjugates; mouse-derived immunoglobulin M (IgM)-biotin (1 μg/well, eBioscience, San Diego, CA) or Le^x-polyacrylamide (PAA)-biotin (1 μg/well, Glycotech, Maryland, CA) was coated onto ELISA plates for investigation of specific binding and the saturated reaction ratio between SA in the conjugates and biotin. The conjugates were added to ELISA plates coated with IgM-biotin or Le^x-PAA-biotin, then incubated at 37° for 30 min. Binding was determined by mouse-derived anti-OVA polyclonal antibody and a horseradish peroxidase-conjugated secondary antibody.

According to the reaction ratio from the above experiments, the purified SA-OVA (4 μg) was mixed with Le^x-PAA-biotin (1 μg) and incubated for 30–60 min at room temperature. The last production, Le^x-OVA, was lyophilized for extended storage. Le^x-OVA contained < 1 ng/20 μg endotoxin.

Cells

Human peripheral blood mononuclear cells (PBMC) were obtained from the whole blood from normal donors over Lymphoprep (Axis Shield, Oslo, Norway). Monocytes were purified from PBMC by adherence for 90 min in complete medium. Non-adherent cells (autologous peripheral blood lymphocytes) were removed gently, washed and frozen as a resource of T cells. The remaining adherent cells (> 90% monocytes by flow cytometry) were induced using 800 U/ml recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF, R & D Systems, Minneapolis, MN) and 1000 U/ml IL-4 (R & D Systems) for 5 days. The immature DCs were obtained and used for internalization assays and antigen-specific T-cell activation experiments.

K562 cell lines transfected with plasmid transiently expressing DC-SIGN-EGFP (K562-ED) were used for the internalization assay of Le^x-OVA.

Internalization assays of ligands

Immature DCs and K562-ED were incubated with ligand or antigen for 60 min at 4°, washed twice and incubated for different times at 37°. Then the cells were harvested and used in a confocal microscopy assay. In brief, the cells were adhered to poly L-lysine-coated glass slides, fixed with 4% polyformaldehyde, stained and analysed using a TCS-NT confocal microscope. In blocking experiments, 20–40 μg/ml anti-DC-SIGN antibody (120507, R & D Systems) was incubated before ligand or antigen treatment for 30 min.

Antigen-specific T-cell sensitization in vitro

Immature DCs were incubated with various antigens (OVA, SA-OVA and Le^x-OVA) for 1 hr at 37° and matured using soluble CD40L (sCD40L, 20 ng/ml, Pepro-

Tech, Rocky Hill, NJ) for a further 24 hr. T cells were isolated from autologous peripheral blood lymphocytes by B-cell negative depletion (R & D Systems). Irradiated (3000 rads) or unirradiated effector DCs were incubated with T cells in the presence of IL-7 (10 ng/ml; day 0), followed by addition of IL-2 (20 U/ml; day 5 for first cycle, day 2 for the other cycles). The ratio of T-cells : DCs was maintained at 10 during the stimulation. IL-2 was added every 3–4 days. The effector T cells were harvested and assayed. The cytokines used in the above experiments were obtained from PeproTech and the OVA (A5378) was from Sigma-Aldrich (St. Louis, MO). In block experiments, anti-DC-SIGN block antibody (40 μg/ml) was incubated with immature DCs 30 min before antigen treatment.

Assays for OVA-specific immune responses

Enzyme-linked immunospot assay (ELISPOT) kits (U-CyTech, Utrecht, the Netherlands) were used to measure antigen-specific interferon-γ (IFN-γ)-producing cell activation according to the manufacturer's protocols. Antigen-specific effector cells were obtained from a 14-day *in vitro* sensitization. Then, the effector T cells were suspended in AIMV serum-free medium (Invitrogen, Carlsbad, CA) and used to detect IFN-γ release, with DC loaded with OVA (20 μg/ml) or without as a specific target. IFN-γ spots were enumerated by a computer-assisted immunospot image analyser.

IFN-γ-producing effector cells were also assayed by intracellular cytokine staining. Briefly, the autologous T cells were cocultured with DCs that were loaded with graded doses of various antigens for 14 days. Then the cells were restimulated with autologous DCs, loaded with OVA (20 μg/ml) and matured with sCD40L (20 ng/ml), for 10 hr in the presence of brefeldin A (5 μg/ml; Sigma-Aldrich) for the last 9 hr. The staining was performed as described elsewhere.[19]

Cytotoxic activity was measured in a standard 4-hr ^{51}Cr-release assay using the OVA-expressing and human leucocyte antigen (HLA) A2.1^{+} breast cancer cell lines MCF-7 as target cells to be killed, as depicted previously.[20] Effector T cells were obtained from four *in vitro* sensitization cycles (one week/cycle). The per cent specific lysis was calculated as follows: % specific lysis = (experimental lysis − minimum lysis)/(maximum lysis − minimum lysis) × 100. Minimum lysis was obtained by incubating the target cells with the culture medium alone. Maximum lysis was obtained by exposing the target cells to 2% Triton X-100 in phosphate-buffered saline.

ELISA assays of cytokines

Supernatants of DC cultures stimulated by Le^{x}-OVA or by Le^{x} oligosaccharide monomer together with sCD40L or ultra-pure LPS (from *Escherichia coli* 0111:B4 strain, InvivoGen, Carlsbad, CA) or not, were harvested after 24 hr, and kept frozen at −70° until use. IL-6, IL-12 (p70), IFN-γ and IL-10 in the supernatant were assayed by ELISA kits from BD PharMingen (San Diego, CA). The minimum detectable dose of IL-10 was 15 pg/ml. The minimum detectable dose of IFN-γ was 7 pg/ml.

The Le^{x} oligosaccharide monomer was synthesized as described briefly: the Le^{x} pentasaccharide was synthesized starting from a protected trisaccharide which we had prepared previously; the structure of the Le^{x} pentasaccharide was fully characterized by [^{1}H, ^{13}C]-nuclear magnetic resonance and mass spectroscopy.

Construct of OVA-expressing plasmid

The *OVA* gene was subcloned into pCI-neo vector (Promega, Madison, WI) using the *Eco*RI and *Sal*I sites. The recombinant plasmid was transfected into MCF-7 with lipofectamine 2000 (Invitrogen, Carlsbad, CA) in combination with CombiMag (OZ Biosciences, Marseille, France). The expression of OVA was confirmed by immunofluorescence.

Statistical analysis

Data are expressed as mean ± SEM. Comparisons were made using Student's *t*-test and analysis of variance (ANOVA). The differences were significant if $P \leq 0{\cdot}05$.

Results

Preparation of OVA conjugated to Le^{x} oligosaccharides

We devised an approach to obtain Le^{x}-OVA antigen. First, OVA were chemically coupled to SA; then, SA-OVA conjugates were linked to Le^{x}-PAA-biotin via SA-biotin binding.

The maleimide-activated whole OVA protein was chemically cross-linked to SA, which had been pretreated with 2-iminothiolane/HCl to produce terminal sulphydryl groups to make coupling easy. The SA-OVA conjugates were verified in SDS–PAGE and Western blot (Fig. 1a–c). According to band density in reference to the SA and OVA, we estimated that the molecular ratio of SA to OVA in the conjugates was 2 : 1. Then, free SA was removed by purifying the conjugates on affinity chromatography for OVA, and unbound OVA smaller than the pore-size rating of 50 000 MW was also removed by ultrafiltration. SA-OVA conjugates were obtained and quantified by Bradford micro-protein assay. The standard curve of OVA protein used for quantification of the conjugates is given in Fig. 1(d).

We then investigated whether SA binding function was affected after coupling to OVA. SA in the conjugates was verified as being able to bind to biotin specifically using

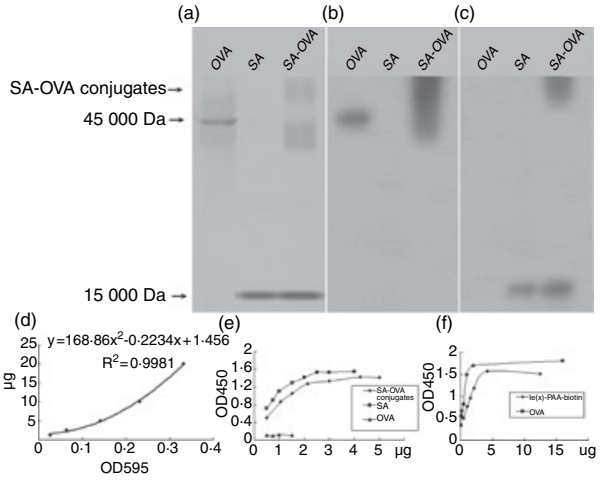

Figure 1. Preparation of Le^x-OVA. (a) OVA, SA and SA-OVA run on SDS–PAGE. The electrophoretic behaviour of the protein (SA-OVA) treated with a chemical cross-linking reagent had changed and displayed a smear band in contrast to the native OVA. Residual OVA (runs a little faster than the native OVA) and SA, in the lane marked SA-OVA, were observed. (b) Immunoblotting with anti-OVA antibody to detect OVA in the SA-OVA conjugates. (c) Immunoblotting with anti-SA antibody to detect SA in the SA-OVA conjugates. (d) The standard curve used for quantification of SA-OVA conjugates in the Bradford micro-assay. (e) SA-OVA conjugates, SA (positive control) and OVA (negative control) were used to detect the specific biotin-binding capacity. (f) Capacity of SA-OVA conjugates to bind Le^x-PAA-biotin.

ELISA in a plate that was coated with mouse-derived IgM labelled with biotin (Fig. 1e). The OVA alone, as a negative control in this experiment, did not show any capacity to bind to biotin. Furthermore, SA-OVA conjugates were also able to bind to Le^x-PAA-biotin, a polyacrylamide polymer of approximately 30 000 MW containing 5%mol biotin and 20%mol Le^x oligosaccharides, coated on the ELISA plate at 1 μg/well, and we estimated that conjugates of approximately 4 μg (about 1·5 μg OVA, according to the OVA standard curve) had complete binding capacity to Le^x-PAA-biotin of 1 μg (Fig. 1f). Therefore, based on the characteristic of SA binding biotin with high affinity, we yielded a targeting to DC-SIGN antigen, Le^x-OVA, through 4 μg : 1 μg reaction ratio.

Specific targeting of Le^x-OVA to DC-SIGN

Previous studies have demonstrated that Le^x oligosaccharide or Le^x-PAA-biotin could target DC-SIGN.[12,13] We further investigated whether Le^x-OVA could target DC-SIGN. To this end, we constructed a transfectant K562-EGFP-DC-SIGN (K562-ED) that expressed the DC-SIGN-EGFP fusion protein. The fusion protein was localized on the cell membrane and was recognized by anti-DC-SIGN antibody (Fig. 2a). As mentioned previously,[12] using Le^x-PAA-biotin, which is a verified specific ligand of DC-SIGN, we confirmed that the fusion protein was able to mediate specific ligand internalization (Fig. 2b). Then we investigated whether Le^x-OVA antigen could be taken up by K562-ED. The K562-ED took up the Le^x-OVA antigen rapidly in 15 min; while the anti-DC-SIGN antibody mostly prevented Le^x-OVA uptake. In contrast, K562-expressing EGFP or K562 cell lines are incapable of Le^x-OVA uptake in 15 min (Fig. 2c–e). This result showed that Le^x-OVA could target to DC-SIGN efficiently.

Primary monocyte-derived DCs were also confirmed to have the capacity for Le^x-OVA uptake in 15 min efficiently, but not for OVA or SA-OVA antigen. Internalization of Le^x-OVA was abrogated significantly when binding of DC-SIGN was blocked (Fig. 2f).

Antigen-specific effector T-cell activation

After obtained the Le^x-OVA antigen, we further investigated the capacity of this novel antigen to induce OVA-specific T-cell activation by assaying the numbers of IFN-γ-producing cells induced by Le^x-OVA or native antigens using an ELISPOT assay and intracellular cytokine staining. We used autologous DCs, pulsed with graded doses of various antigens and matured with sCD40L, as a stimulator to activate effector T cells. After 14 days of *in vitro* sensitization, IFN-γ-producing cells were detected in response to the specific target cells, which were autologous DCs loaded with OVA (20 μg/ml) and matured with sCD40L. As shown in Fig. 3(a) the T cells spontaneously produced some spots in the presence of autologous mature DCs unloaded by antigen (mixed lymphocyte reaction $410 \pm 76{\cdot}34$ spots/10^6 T cells), but spots were rarely seen in the presence of autologous mature DCs alone. Compared to ELISPOTs from mixed lymphocyte reactions, at concentrations ≤ 1 μg/ml (especially 0·01 μg/ml), Le^x-OVA invoked significant numbers of IFN-γ-producing cells ($P < 0{\cdot}01$ for 0·01–1 μg/ml group); SA-OVA or OVA, however, did not generate significant OVA-specific IFN-γ-producing cells. The blockade of anti-DC-SIGN antibody reduced IFN-γ-producing cell numbers induced by Le^x-OVA to approximately the level of the mixed lymphocyte reaction. Moreover, in the absence of the specific target cells, the numbers of IFN-γ-producing cells in effector T cells were not significantly different between the Le^x-OVA and control groups (Fig. 3b).

The data from intracellular IFN-γ staining also showed similar results. IFN-γ^+ CD8$^+$ T cells were induced by doses of Le^x-OVA between 0·01 and 10 μg/ml; however, ≤ 1 μg/ml SA-OVA or OVA could not activate effector T cells ($P < 0{\cdot}01$ for 0·01–1 μg/ml group) (Fig. 3c). It was indicated that Le^x-OVA promoted the activation of OVA-specific IFN-γ-producing cells, and 0·01 μg/ml of Le^x-OVA induced balanced numbers of OVA-specific

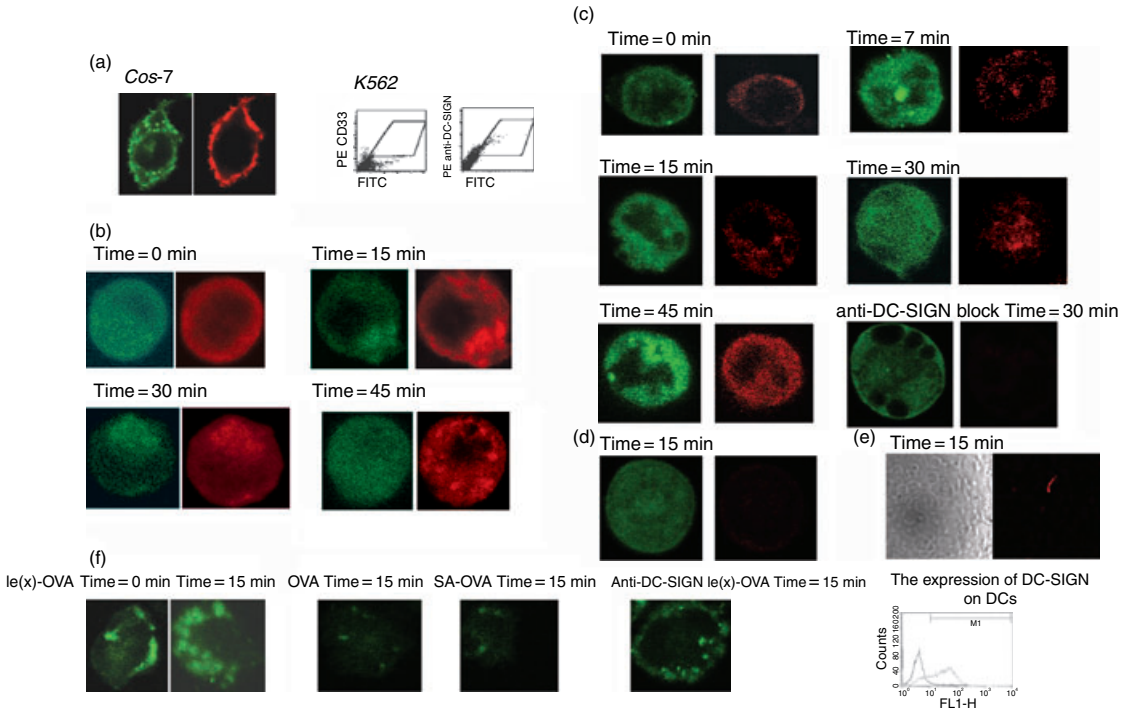

Figure 2. Le^x-OVA was specifically targeted to DC-SIGN. (a) Left panel: expression of DC-SIGN-EGFP (green) fusion protein was assayed on Cos-7 cell lines by confocal microscopy. The fusion protein was stained with anti-DC-SIGN(Cy3). Right panel: expression of DC-SIGN-EGFP (green) fusion protein on K562 cell lines was detected by double colour in a fluorescence-activated cell sorter assay. The phycoerythrin (PE) signal was from anti-DC-SIGN or anti-CD33 (a molecule expressed on the K562 cell lines used to gate). (b) Le^x-PAA-biotin uptake by K562-ED. The cell was stained with streptvidin-Cy3 for localization of Le^x. (c) Le^x-OVA uptake by K562-ED. The cell was stained with rabbit-derived anti-OVA and goat anti-rabbit IgG(Cy3) for localization of OVA. (d) Le^x-OVA was not taken up by K562 cell lines expressing EGFP in 15 min. (e) Le^x-OVA was not taken up by K562 cell lines in 15 min. (f) Le^x-OVA uptake by the primary monocyte-derived DCs. The expression of DC-SIGN on the DCs is also shown.

IFN-γ-producing cells compared to >1 μg/ml of OVA alone. Because approximately 0·0033 μg of OVA alone could be obtained from 0·01 μg of Le^x-OVA, the DC-SIGN receptor-targeted antigen was at least 300-fold more effective for activation of effector T cells, including $CD8^+$ T cells, than the non-targeted antigen.

The HLA-$A2.1^+$ MCF-7 cell line expressing OVA protein as a specific target (Fig. 4a) triggered the cytotoxic T-cell response (Fig. 4b,c). Cytolysis induced by Le^x-OVA (10 μg/ml) was two-fold higher than that induced by OVA alone ($P < 0·01$). However, no significant cytotoxic T-cell response was shown by MCF-7 that did not express OVA protein.

Cytokine secretion and DC maturation induced by Le^x oligosaccharide clusters

To investigate whether interaction between Le^x oligosaccharides and DC-SIGN results in potential immunoregulatory signals, we assayed the secretion of cytokines in the cell supernatant of DCs treated with OVA or Le^x-OVA. There was no significant distinction between IFN-γ levels in supernatants of different groups (Fig. 5a); however, the secretion of IL-10, a mediator of immune suppression in ManLAM-treated DCs, as reported previously,[9] increased significantly in the group given Le^x-OVA together with ultra-pure LPS (Fig. 5a,b) ($P < 0·01$ for 1–100 ng/ml LPS group). In the supernatants of DCs treated with Le^x-OVA alone and of DCs treated with Le^x-OVA in combination with sCD40L, IL-10 was not increased (Fig. 5a,b). We further investigated the secretion of IL-6 in the supernatant of DCs that were treated with Le^x-OVA together with doses of ultra-pure LPS from 1 ng/ml to 100 ng/ml, and little increase of IL-6 level was observed (Fig. 5c). The level of IL-12p70 was hardly detectable. Interestingly, the anti-DC-SIGN blocking antibody (507) did not block the secretion of IL-10. Instead, as for Le^x sugar, it promoted the secretion of IL-10 together with ultra-pure LPS ($P < 0·01$) (Fig. 5b,c).

Different forms of the Le^x oligosaccharides assembly had a different effect on the secretion of IL-10. Unlike Le^x oligosaccharides, clusters existed in Le^x-OVA; 10 μg/ml Le^x oligosaccharide monomer, even when combined with ultra-pure LPS, was not observed to promote secretion of IL-10 (Fig. 5d).

We also examined if the interaction between Le^x oligosaccharide clusters and DC-SIGN would result in the suppression of the maturation of DCs. It was shown that

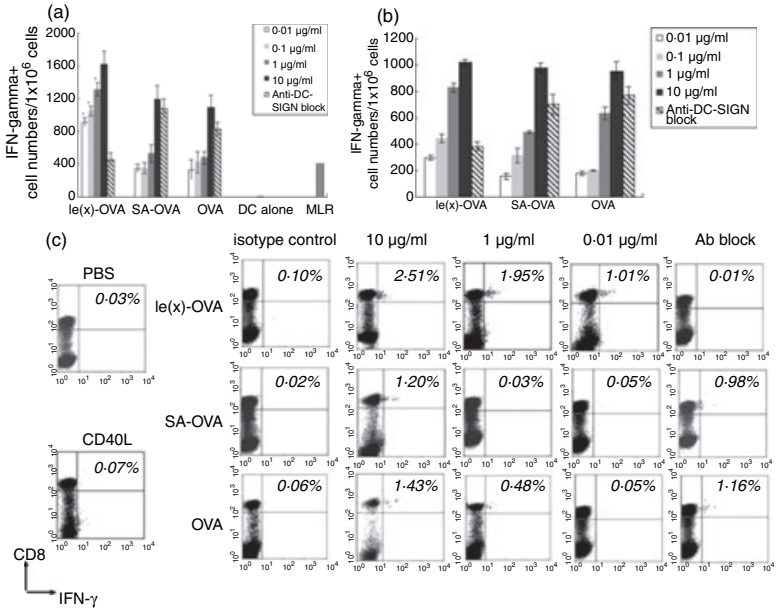

Figure 3. Antigen-specific IFN-γ-producing cells increased after DC-SIGN targeting via Le^x oligosaccharides. (a) Effector T cells were generated from *in vitro* sensitization for 14 days and were used to detect the numbers of IFN-γ-secreting cells. DCs loaded with OVA and matured with sCD40L were added to wells to be a specific target; 40 μg/ml anti-DC-SIGN was used for blockade in the 10 μg/ml Le^x-OVA stimulation group. Data are representative of three independent experiments performed in triplicate with similar results. (b) Effector T cells were stimulated and assayed as above, except without specific target cells added into the ELISPOT assay wells. Controls (not shown): lane 1, positive control (autologous PBMC were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml)): 140 ± 47·15 spots/10^5 PBMC; lane 2, negative control (autologous PBMC): 0–2 spots/10^5 PBMC; lane 3 negative control (autologous PBMC with OVA 20 μg): 4–6 spots/10^5 PBMC. Data are representative of three independent experiments performed in triplicate with similar results. (c) The antigen-specific IFN-γ-producing CD8$^+$ T cells were observed. Effector T cells were stimulated as above; 40 μg/ml anti-DC-SIGN was used for blockade in the 10 μg/ml Le^x-OVA stimulation group. Data are representative of four independent experiments performed with similar results. Statistical significance of the differences ($P < 0·01$) is between 0·01 and 1 μg/ml of Le^x-OVA and other controls (SA-OVA and OVA alone) stimulation groups. Asterisks indicated statistical significance ($P < 0·01$, ANOVA).

with the increase of ultra-pure LPS from 1 to100 ng/ml, pretreatment by Le^x-OVA could not alter the growth rate of the mean fluorescence intensity of CD86 induced by LPS (Fig. 5e). It was suggested that DCs treated with Le^x oligosaccharide clusters did not display significant maturation suppression.

Discussion

DC-SIGN has been characterized by its efficient capture of pathogens and restricted expression on DCs, which makes it a preferable candidate for a vaccine-targeting receptor.[11] Many natural ligands of DC-SIGN expressed on pathogens have been identified. Some natural ligands might subvert DC functions to help the pathogens escape immune attack, such as ManLAM from *Mycobacterium tuberculosis*, which implies that DC-SIGN in some circumstances may be a disadvantage in the induction of antigen-specific immunity. Thus, selection of proper targeting strategies is critical. A recent study by Tacken *et al.* has verified that targeting of DC-SIGN mediated by an anti-DC-SIGN antibody displays more efficient antigen presentation than a native antigen.[21] In the present study, we demonstrated that the targeting of DC-SIGN mediated by Le^x oligosaccharides also enhanced T-cell immune response.

IFN-γ is a representative effector cytokine of CD8$^+$ T cells, and in the presence of specific targets an assay of CD8^{high} IFN-γ-producing cells would indicate the activation of antigen-specific effector CD8$^+$ T cells. Antigen-specific lysis is another important function of effector CD8$^+$ T cells. The results from ELISPOT, intracellular IFN-γ staining and ^{51}Cr-release assays demonstrated that Le^x oligosaccharides targeting of DC-SIGN promoted antigen-specific CD8$^+$ T-cell activation, which was at least 300-fold more efficient than pulsing DCs with non-targeted antigen. Blockade of binding to DC-SIGN decreased the numbers of antigen-specific IFN-γ-producing cells significantly, which indicated that internalization mediated by DC-SIGN played an important role in antigen-specific T-cell activation. Internalization mediated by DC-SIGN might result in antigen entry into a specific and efficient presentation pathway, as mentioned in the DEC-205 targeting study,[22,23] which promotes the production of antigen peptide–major histocompatibility complex complexes and the subsequent activation of effector

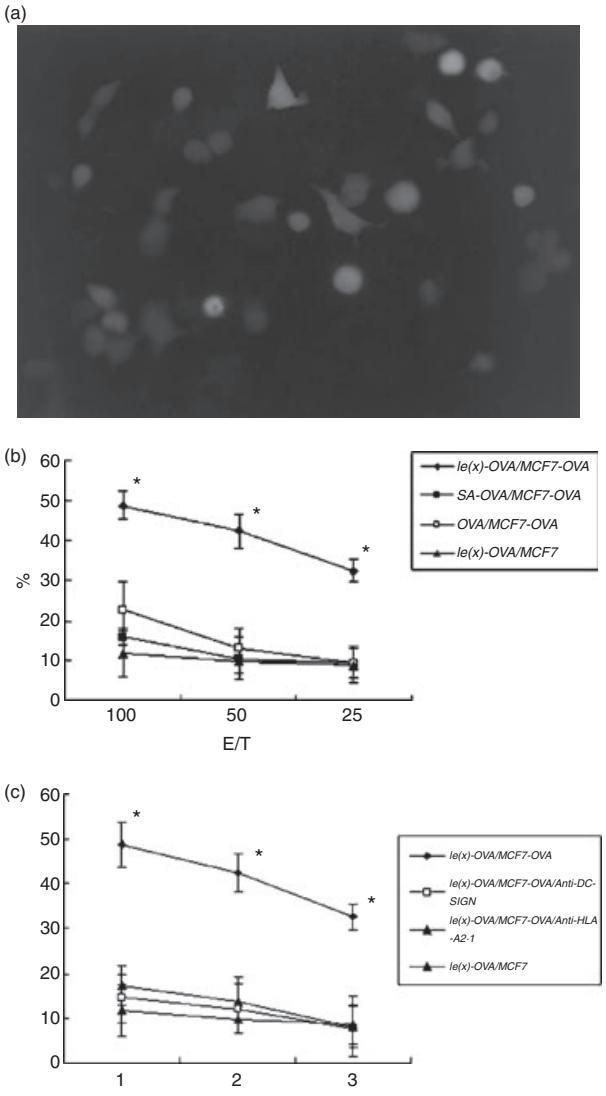

Figure 4. Le^x-OVA enhanced antigen-specific cytotoxic response. (a) The expression of OVA was displayed after transfection with *OVA* gene plasmid for 24 hr in the MCF-7. (b and c) Specific lysis was measured with ^{51}Cr-release assay. Effector T cells were stimulated with autologous DCs (irradiated) loaded with various antigens at doses of 10 μg/ml for 4 weeks. Then the effector T cells were used to assay antigen-specific lysis. Data are representative of three independent experiments performed in triplicate with similar results. Asterisks indicate statistical significance ($P < 0{\cdot}01$, ANOVA).

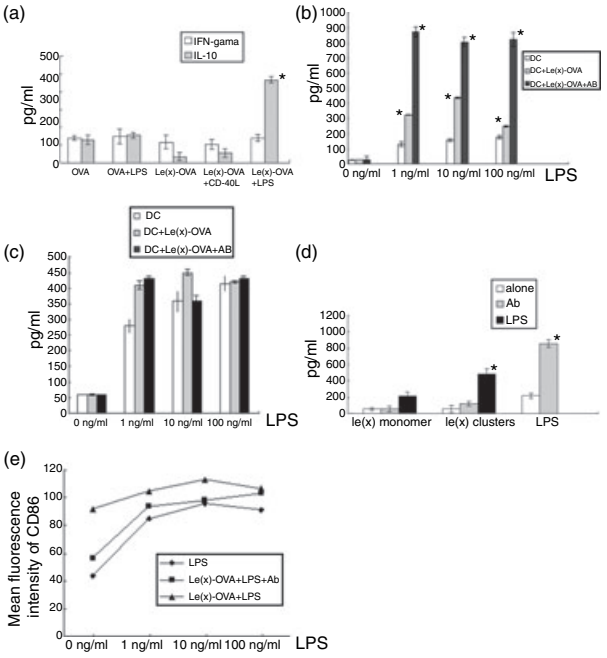

Figure 5. Le^x-OVA together with ultra-pure LPS instead of sCD40L promoted the secretion of IL-10. (a) Le^x-OVA (10 μg/ml) together with LPS promoted the secretion of IL-10 on DCs. The supernatants of DCs treated differently were used to assay levels of IL-10 and IFN-γ. Data are representative of three experiments. (b) Secretion of IL-10 was examined on the supernatants of DCs treated by Le^x-OVA together with graded doses of ultra-pure LPS. The block antibody (40 μg/ml) could not prevent but enhanced the secretion of IL-10. Data are representative of three independent experiments in triplicate. (c) As above, secretion of IL-6 was assayed. Data are representative of three independent experiments in triplicate. (d) Le^x oligosaccharides monomers (10 μg/ml) together with ultra-pure LPS (10 ng/ml) could not increase the level of IL-10. However, the blocking antibody (20 μg/ml) still enhanced the secretion of IL-10. Data are representative of three independent experiments. (e) Le^x-OVA engagement to DC-SIGN did not decrease the expression of CD86 by flow cytometry. Data are representative of two experiments. Asterisks indicate statistical significance ($P < 0{\cdot}01$, Student's *t*-test).

T cells. The sCD40L was an appropriate immune adjuvant to promote CD8$^+$ T-cell responses, not to increase the level of IL-10 and inhibit the maturation of DCs, after Le^x oligosaccharides targeting to DC-SIGN.

IL-10 is one of the agents known to be critical for the induction of immune tolerance,[24–27] and it has been reported to inhibit the maturation of DCs and the maturation-driven shift from inflammatory chemokine receptors to the lymphoid homing receptor CCR7, which stops DCs from migrating to the lymph nodes for recruitment of T cells and contributes to the generation of antigen-specific T-cell anergy.[25–27] IL-10 is thought to be an anti-inflammatory cytokine for the limitation of excessive inflammatory reactions in response to LPS and an inhibitor of T-cell-specific activation.[28,29] Thus, IL-10 would affect the result of antigen-specific T-cell immune responses, either activation or suppression.

Our results indicated no increase of IL-10 in the DC groups given Le^x oligosaccharides alone or together with sCD40L. However, together with ultra-pure LPS, Le^x oligosaccharide clusters significantly promoted the secretion of IL-10, while that of IL-6 was little changed. It is known that no C-type lectin other than DC-SIGN expressed on human DCs has been identified as recognizing Le^x oligosaccharides,[10] and that ultra-pure LPS is

recognized by Toll-like receptor 4 (TLR4) on DCs,[30] which strongly suggests that collaboration occurs between DC-SIGN and TLR4. One of the mouse homologues of human DC-SIGN, mSIGNR1,[31,32] is also found to have a collaborative effect with TLR4 to enhance signal transduction by recognition of LPS.[33] Mice with mSIGNR1 knock-out are significantly more susceptible to *Streptococcus pneumoniae* infection and fail to clear *S. pneumoniae* from their circulations.[34] Both our data and those of others suggested that the collaboration between TLR4 and DC-SIGN was involved in the control of IL-10 secretion, which implies a regulation mechanism of innate and adaptive immune responses to antigen targeting of DC-SIGN. Future study is needed to investigate the effect of LPS on antigen-specific immunity after Le^x oligosaccharide targeting of DC-SIGN. However, this was beyond the scope of this paper.

Not only different immune adjuvants but also different assemblies of Le^x oligosaccharides could result in a different level of IL-10. The Le^x oligosaccharide monomer together with LPS did not promote the secretion of IL-10, although the dose of Le^x monomer was 10 times more than the 10 μg/ml Le^x-OVA used (data not shown) that caused a notable increase in IL-10. It is possible that the interaction energy between different assembly forms of Le^x oligosaccharides and DC-SIGN results in these distinct effects.[35] The interaction between the Le^x oligosaccharide clusters that exist in Le^x-OVA and DC-SIGN seemed to promote the secretion of IL-10 more easily. More direct results are needed to verify the hypothesis.

In conclusion, this study demonstrated: (1) that Le^x oligosaccharides targeting of DC-SIGN would induce an at least 300-fold more efficient T-cell immune response; (2) that targeting mediated by Le^x oligosaccharides may be a novel appropriate strategy for the targeting of DC-SIGN for vaccination immunity; (3) that an appropriate immune adjuvant, which may decide the form of immune response, needs to be tested as one of the components of future vaccines involved in DC-SIGN targeting.

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