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Targeted Delivery of Mycobacterial Antigens to Human Dendritic Cells via Siglec-7 Induces Robust T Cell Activation

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Lipids from mycobacteria can be presented to human T cells by group 1 CD1 Ag-presenting molecules (CD1a, CD1b, and CD1c). Group 1 CD1-restricted T cells are activated by lipid Ags presented by myeloid dendritic cells (DCs), after which they generate antibacterial effector functions, including IFN-γ secretion and cytosis. Thus, mycobacterial lipids are being investigated as components of novel vaccines for mycobacterial infections. In this study we show that the mycobacterial lipid Ag C80 glucose-6-monomycolate can be delivered to human CD1b+ DCs via targeted liposomal nanoparticles, leading to robust group 1 CD1-restricted activation of T cells. Targeting was achieved by decorating the liposomes with a high-affinity glycan ligand of sialic acid–binding Ig-like lectin (Siglec)-7, a siglec receptor expressed on DCs that mediates rapid endocytosis and transport of its cargo to lysosomes. An Ab to Siglec-7 completely blocked the binding of targeted liposomes to human monocyte-derived DCs (Mo-DCs), demonstrating their targeting specificity. Mo-DCs pulsed with targeted liposomes containing C80 glucose-6-monomycolate more potently activated a CD1b-restricted T cell line relative to Mo-DCs pulsed with free lipid Ag or antigenic liposomes without Siglec-7 ligand. These data suggest that the endocytic function of Siglec-7 can be exploited to deliver glycolipid Ags to their target cell and increase the efficiency of display to T cells. The Journal of Immunology, 2014, 193: 1560–1566.

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Abbreviations used in this article: DC, dendritic cell; GEM, germlin-encoded mycolyl lipid–specific; GMM, glucose-6-monomycolate; Mo-DC, monocyte-derived DC; PEG-DSPE, polyethylene glycol–distearoyl phosphoethanolamine; siglec, sialic acid–binding Ig-like lectin.

Glucose-6-monomycolates (GMMs), which have acyl chains attached to a glucose head group, are abundant lipid components present in the cell wall of all mycobacterial species studied to date. They bind to CD1b by their acyl chains, and although the acyl chains of GMMs vary by mycobacterial species, they are all completely buried in the lipophilic groove of CD1b (14). As a result, the glucose head group is exposed as a common antigenic epitope (14). Accordingly, T cells that recognize GMM from one source as their matched Ag also react to GMM from other sources (9). Furthermore, animal studies suggest that GMM is an immunodominant Ag during natural infection (11, 15), and recent studies with CD1b tetramers prove that polyclonal populations of GMM-reactive T cells exist in human tuberculosis patients (4, 7). Of note, conserved germline-encoded, mycolyl lipid–specific (GEM) T cells have been identified as high-affinity responders to GMM in humans (7). Whereas GMM-specific T cells and GEM T cells are found at a low frequency in healthy individuals (0.002%), their expansion is commonly observed in active and latent tuberculosis infection, accounting for 0.01% of T cells (4, 7, 16). Additionally, a second type of polyclonal GMM-reactive T cell type is known as LDN5-like T cells. LDN5-like T cells are so named because they express TCRs and cytokine patterns that are similar to those associated with a T cell clone named LDN5 (17). GEM T cells are defined by high-affinity TRAV1-2* TCRs, whereas TRBV4-1* LDN5-like T cells have intermediate affinity for CD1b and GMM (7, 17). Following Mycobacterium bovis bacillus Calmette–Guérin vaccination, GMM-reactive T cells produce IFN-γ and TNF in a CD1b-restricted manner (6). Therefore, vaccination activating GMM-reactive T cells are now being studied as a new method to alter immunity to M. tuberculosis in vivo.

Current tuberculosis vaccine trials focus on mycobacterial protein subunit vaccines, and antigenic lipids including GMM are thought to comprise a next generation of vaccine candidates (18). To enhance the presentation of GMM, targeted delivery to the CD1b+ APCs is desired, because the cellular expression of CD1b is restricted to certain cell types. In the periphery, CD1b is exclusively expressed on DCs in healthy individuals (19) as well as patients with Mycobacterium leprae infection (20). Thus, as is also the case...
for MHC class I and II, myeloid DCs are thought to be the main functionally important APC in the periphery (21). For DC-targeted Ag delivery, Abs toward the cell surface receptors have been investigated for delivery of protein Ags conjugated to the Ab, some of which have been in human clinical trials for tumor and HIV vaccines (22, 23). However, more suitable delivery platforms for hydrophobic lipid Ags are yet to be developed and tested. Previously we have developed a targeting platform based on liposomal nanoparticles bearing glycan ligands of sialic acid–binding Ig-like lectins (siglec) capable of in vivo delivery of both hydrophilic and hydrophobic agents to siglec-expressing immune cells (24–27). Siglecs are a cell surface lectin family that recognizes sialic acids as ligands and are expressed on human leukocytes in a cell type–restricted manner (28–30). Among human siglecs, Siglec-7 is expressed on DCs as well as on other human leukocytes, including NK cells, neutrophils, monocytes, and macrophages (30–32). Based on the restricted expression of Siglec-7, it has been proposed as an attractive target for cell-targeted therapies directed to myeloid cells (29, 33). We have recently developed a glycan ligand of high affinity and selectivity for Siglec-7 suitable for use for targeting cells expressing this siglec (34).

In this study, we investigated the potential for efficient delivery of GMM to CD1b+ human monocyte-derived DCs (Mo-DCs) using anti-Siglec liposomes bearing ligands of Siglec-7. We found that targeted liposomes were captured by Mo-DCs and delivered to lysosomes in a Siglec-7–dependent manner. Mo-DCs pulsed with targeted liposomes containing C80 GMM, a GMM with long acyl chains (4, 14), potently activated the CD1b-restricted human T cell line LDN5, shown by IFN-γ production. Thus, we conclude that the Siglec-7 endocytic pathway can be exploited for targeted delivery of mycobacterial lipid Ags to human DCs.

Materials and Methods

Cells

CD1b-reactive human T cell line LDN5 cells were originally derived from a human skin lesion of a leprosy patient and maintained as described (9), and this T cell clone expresses a TRBV4-1* TCR that is representative of a natural T cell population known as LDN5-like T cells (17). Human Mo-DCs were generated from the culture of peripheral blood monocytes in vitro. Briefly, monocytes were enriched with CD14 magnetic beads (BD Biosciences) fromuffy coats from peripheral blood obtained from healthy human donors according to the Institutional Review Board at The Scripps Research Institute. After informed consent, 50 ml blood was collected from asymptomatic donors. Monocytes were isolated as described (35). Ficoll-Paque Plus (GE Healthcare) was used for the density gradient centrifugation to separate PBMCs. A human IFN-γ ELISA kit (BioLegend) was used to measure human IFN-γ.

Liposomes

The liposomes were prepared as described (34). Briefly, 4 mol% Siglec-7–targeted liposomes were composed of distearoyl phosphatidylcholine (Avanti Polar Lipids)/cholesterol (Sigma-Aldrich)/polyethylene glycol-diestearoyl phosphoethanolamine (PEG-DSPE; NorPe Corporation)/Siglec-7 ligand-lipid (34) in a 58:37:1:4 molar ratio. Naked liposomes were composed of 5 mol% PEG-DSPE instead of the Siglec-7-ligand-anti-Siglec-7 targeted liposomes substituted 1 mol% distearoyl phosphatidylcholine for 1 mol% C80 GMM. Fluorescent liposomes contained 0.2 mol% of Alexa Fluor 647–PEG-DSPE (27). For liposome preparation, lipid components in dimethyl sulfoxide (Siglec-7-ligand-lipid, C80 GMM, and Alexa 647–PEG-DSPE) were mixed and lyophilized in a glass tube. The other components in chloroform were then added to the tube and dried completely by airflow. The dried lipids were hydrated with 1 ml PBS, sonicated, and then extruded by a liposome extruder (Avanti Polar Lipids) until the size became ~100 nm as measured by a Zetasizer (Malvern). The GMM concentration in the antigenic liposomes was 10 μM.

Flow cytometry

Cells were washed with HBSS containing 0.1% BSA, 2 mM EDTA, and 0.1% Na3EDTA (FACS buffer), blocked with anti-human CD32 (3D3, BD Biosciences) for 5 min at 25˚C, and stained with Abs for 30 min at 4˚C. Stained cells were washed once with FACS buffer and analyzed by a FACS Calibur or LSR II (BD Biosciences). Propidium iodide (1 μg/ml) was added to the sample before the analysis for exclusion of the dead cells. Acquired data were analyzed with FlowJo (Tree Star). For the liposome binding analysis, cells were incubated with the liposomal GMM at 37˚C and then washed and analyzed as above. To assess the Siglec-7 dependence in the liposome binding, cells were first incubated with 10 μg/ml anti-Siglec-7 or mouse IgG1 isotype control Ab (BioLegend) for 30 min at 4˚C followed by the addition of the liposomes.

LDN5 cell activation

Mo-DCs were pulsed with indicated reagents for 20 min at 37˚C in the sterile FACS buffer and washed. To assess the Ag presentation activity of the cells, 5 × 104 Mo-DCs were cultured with 5 × 105 LDN5 cells. For the comparison of liposomal and free C80 GMM, the same number of LDN5 cells was cultured with 2.5 × 105 Mo-DCs. After 20 h, the culture supernatants were collected and human IFN-γ was measured by ELISA. To assess the targeting specificity of the liposomes, Mo-DCs were first incubated with 10 μg/ml anti–Siglec-7 or isotype control Ab for 30 min at 4˚C prior to the addition of the liposomes. To determine Siglec-7 dependence in the LDN5 activation, 10 μg/ml anti–CD1b Ab was added to the culture. For some experiments, Mo-DCs mixed with PBMCs were pulsed as described above. The Mo-DCs were isolated by CD209 magnetic beads (Miltenyi Biotec) and used for the Ag presentation assay.

Microscopy

Mo-DCs on the coverslip were incubated with 100 μM Alexa Fluor 647–labeled Siglec-7–targeted or naked liposomes in the culture medium for 90 min at 37˚C. The cells were washed with PBS, fixed with 4% PFA in PBS for 5 min at 25˚C, and permeabilized with PBS containing 0.1% saponin and 0.1% BSA for 5 min at 25˚C. The cells were then blocked with the culture medium for 1 h at 25˚C and stained with 1 μg/ml anti-LAMP1 and EEA-1 in the culture medium for 18 h at 4˚C. The cells were washed with PBS and stained with 1 μg/ml anti-rabbit IgG–Alexa Fluor 555 (Life Technologies) in the culture medium containing 1 μg/ml DAPI for 30 min at 25˚C. The cells were washed with PBS and mounted with anti-fade mounting solution (Life Technologies). Images were obtained on a Bio-Rad (Zeiss) Radiance 2100 Rainbow laser scanning confocal microscope. The acquired images were analyzed using the ImageJ processing program (Wright Cell Imaging Facility at University Health Network, Toronto, ON, Canada). The degree of colocalization between Siglec-7–targeted liposome and LAMP-1 or EEA-1 was evaluated using the intensity correlation analysis plugin of the colocalization module of the program (36). Analysis was conducted on three individual cells, each showing good expression of both Abs from different images. The calculated Pearson correlation coefficients (r) obtained from each cell were averaged and are expressed as means ± SD.

Statistical analysis

A Student t test was used for statistical analysis on Prism software (GraphPad Software). A p value <0.05 was considered statistically significant.

Results

Siglec-7–targeted liposomes bind specifically to Siglec-7 on human Mo-DCs

Recent screening of a sialic acid analog glycan library identified a high-affinity glycan ligand for Siglec-7, and liposomes bearing...
this ligand specifically bound to Siglec-7–bearing cells, but not to
cells bearing any other human siglec (Fig. 1A) (34). To test whether
these Siglec-7–targeted liposomes bound to human DCs via Siglec-7,
we assessed the binding of Alexa Fluor 647–labeled liposomes to
human Mo-DCs, which express Siglec-7 as well as the Ag-presenting
molecule CD1b (Fig. 1B). As shown in Fig. 1C, the Alexa Fluor 647–labeled
Siglec-7–targeted liposomes bound to Mo-DCs, whereas
“naked” untargeted liposomes did not. This binding was completely
Siglec-7–dependent because anti–Siglec-7 Ab abolished the binding
of the liposomes (Fig. 1D). Although other siglecs, including Siglec-1,
-3, -5, and -9, are known to be expressed on Mo-DCs
(Supplemental Fig. 1) (32), these data demonstrate that Siglec-7–
targeted liposomes specifically target Siglec-7 expressed on Mo-DCs.

Siglec-7–targeted liposomes are delivered to lysosomes in
Mo-DCs
Next, we asked whether the Siglec-7–targeted liposomes were
internalized by Mo-DCs upon binding to Siglec-7. We incubated

FIGURE 1. A glycan ligand-decorated liposome targets to Siglec-7 on human DCs. (A) Structure of the Siglec-7–targeted liposomes. (B) Expression of Siglec-7 and CD1b on human Mo-DCs. Human Mo-DCs were generated by in vitro culture of peripheral blood monocytes. The cells were stained with anti–Siglec-7 and
CD1b (filled histogram) or isotype control Abs (open histogram), then washed and analyzed by flow cytometry. (C) Alexa Fluor 647–labeled Siglec-7–targeted liposomes bind to Mo-DCs. Mo-DCs were incubated with 10 μM targeted liposomes (gray, filled), naked liposomes (broken line), or buffer only (control, solid line). Cells were washed and analyzed by flow cytometry. (D) The binding of Siglec-7–targeted liposomes to the Mo-DCs is Siglec-7–dependent. Mo-
DCs were first incubated with 10 μg/ml of the indicated Abs or buffer only for 30 min at 4˚C and then incubated with the liposomes (10 μM) or buffer only (control). The cells were washed and analyzed by flow cytometry. The binding of liposomes is shown as mean fluorescence intensity (MFI). Error bars indicate SD. ***p < 0.001, by Student t test. Data show the results of one out of two experiments with similar results.

FIGURE 2. Siglec-7–targeted liposomes are delivered to lysosomes in Mo-DCs. Mo-DCs adhered on a cover slip were incubated with 100 μM Alexa Fluor 647–labeled Siglec-7–targeted or naked liposomes for 90 min at 37˚C. Cells were then washed, fixed, and permeabilized. The permeabilized cells were stained with anti-LAMP1 (A) and
EEA-1 (B) to visualize lysosomes and early endosomes, respectively. Data show representative results of two experiments.
Mo-DCs with Alexa Fluor 647–labeled liposomes for 90 min at 37°C. As shown in Fig. 2A, Siglec-7–targeted liposomes showed a punctate staining pattern, typical for the staining of intracellular small vesicles, and they were partially colocalized with the lysosomal marker LAMP-1 ($r = +0.35 \pm 0.09$, Fig. 2A). In contrast, little or no binding of naked liposomes was detected ($r = +0.06 \pm 0.02$, Fig. 2A). We also observed a colocalization of Siglec-7–targeted liposomes with the early endosomal marker EEA-1 to a similar extent ($r = +0.32 \pm 0.04$, Fig. 2B). These data confirm that Siglec-7 is an endocytic receptor (33) and is capable of delivering ligand-decorated liposomes to lysosomes via an endocytic pathway involving early endosomes.

Siglec-7 endocytic pathway leads to lipidAg presentation to CD1b-restricted T cells

Because the mycobacterial lipid Ag C80 GMM has been shown to be loaded onto CD1b in acidic lysosomal compartments (37), we hypothesized that targeted delivery of antigenic liposomes to lysosomes of DCs would lead to presentation of C80 GMM to CD1b-restricted T cells (Fig. 3A). To test this, we incorporated C80 GMM into the Siglec-7–targeted liposomes or naked liposomes. Mo-DCs were then pulsed with liposomes containing C80 GMM, washed, and cultured with the human CD1b-restricted T cell clone LDN5 to assess the Ag-presenting activity. The LDN5 reporter cell line was used because it has a type of TCR expressed by a recently discovered population of polyclonal T cells named after this cell line, known as LDN5-like T cells (17). Ag presentation assays using primary polyclonal CD1b-restricted T cells can be confounded by concurrent allogeneic responses to MHC unmatched DCs, whereas LDN5 clone sensitively detects CD1b/GMM complexes on MHC unmatched primary Mo-DCs used in these assays (4, 7). Also, in attempts to conduct Ag presentation using polyclonal peripheral blood T cells obtained from tuberculosis patients, high background responses of non–Ag-specific T cells in the presence of medium alone obscured the GMM-specific response (data not shown). However, the LDN5 T cell clone, which was originally derived from a leprosy patient (9) and has been used extensively as an in vitro reporter for human CD1b-restricted T cells (4, 9, 13, 37), overcame both technical limitations. Upon GMM recognition, the LDN5 T cell clone produces IFN-$\gamma$, which is a common CD1b-restricted T cell response seen in tuberculosis patients, polyclonal LDN5-like T cells, and bacillus Calmette–Gue´rin-vaccinated healthy individuals (4–7, 17). As shown in Fig. 3B, Mo-DCs pulsed with Siglec-7–targeted liposomal C80 GMM induced significantly more IFN-$\gamma$ from LDN5 cells than did those pulsed with naked liposomal C80 GMM. This activation was abolished by the treatment of Mo-DCs with anti–Siglec-7 Ab prior to the addition of the liposomes (Fig. 3C). As a further control, addition of anti-CD1b blocking Ab into the coculture of Mo-DCs with the T cells inhibited this activation (Fig. 3C). Taken together, these data with the LDN5 clone clearly demonstrate that the Siglec-7 endocytic pathway is able to effectively deliver lipid Ag for presentation to CD1b-restricted T cells.

Delivery of the mycobacterial Ag to DCs via Siglec-7 enhances CD1b-restricted T cell activation

To assess the efficiency of Siglec-7–mediated delivery of lipid Ags we compared the ability of Mo-DCs pulsed with 1 $\mu$M C80 GMM supernatants was measured as above. **p < 0.01, ***, p < 0.001, by Student $t$ test. Data show the results of one out of two experiments with similar results.
in targeted antigenic liposomes or the free lipid to activate CD1b-restricted T cells. Mo-DCs pulsed with Siglec-7–targeted liposomal C80 GMM consistently activated LDN5 cells more efficiently than did those pulsed with free C80 GMM, with Mo-DCs prepared from blood of five human donors (Fig. 4A). Titration of C80 GMM showed that Siglec-7–targeted liposomal Ag produced equivalent activation of LDN5 cells at a 10-fold lower dose than free C80 GMM (Fig. 4B).

Because other leukocytes express Siglec-7, such as NK cells and monocytes, we sought to determine whether a mixed population of cells expressing Siglec-7 would impact presentation by Mo-DCs. To this end we mixed the Mo-DCs with PBMCs and incubated the cell mixture with the liposomal C80 GMM. Mo-DCs as well as non–DC populations were then separated and cocultured with LDN5 cells to test Ag-presenting activity. We found that DCs isolated from the cell mixture (Supplemental Fig. 2) activated LDN5 cells whereas remaining non-DCs did not support activation, confirming the unique ability of DCs to present Ag and demonstrating robust Siglec-7–mediated targeting to DCs in a mixed population of cells (Fig. 4C).

**Discussion**

Several vaccination studies have assessed the ability of mycobacterial lipids, including GMM formulated in liposomes, to generate T cell responses in vivo (12, 38). In the guinea pig model of tuberculosis, animals vaccinated with bacterial lipid extracts showed reduced bacterial burden and pathology in the lung (38), which is presumed to be due to activation of CD1-restricted

**FIGURE 4.** Siglec-7–mediated delivery of GMM induces robust CD1b-restricted T cell activation. (A) Siglec-7 liposomal C80 GMM activates LDN5 cells more efficiently than do free Ags. Mo-DCs were incubated with 1 μM C80 GMM formulated in naked and targeted liposomes or buffer only (untreated). The cells were washed and cultured with LDN5 cells. After 20 h, IFN-γ in the culture supernatants was measured by ELISA. Data show the results using Mo-DCs obtained from five different donors. (B) Siglec-7 liposomal C80 GMM is ∼10-fold more effective than free Ag. Mo-DCs were treated with indicated GMM reagents and LDN5 cell activation was analyzed as in (A). Note that naked and free lines overlap. (C) Siglec-7–targeted liposomes deliver C80 GMM to human DCs in the mixed cell population. Mo-DCs were mixed with PBMCs and incubated with the reagents as in (A). DCs were then isolated by anti-CD209 magnetic beads, and DCs and non-DCs were tested for Ag-presenting activity. ***p < 0.001, by Student t test. Data show the results of one out of two experiments with similar results.
T cells based on mechanistic studies with the same vaccine (12). Although a detailed analysis of the cell types that captured Ag from the liposomal vaccine was not performed, it is notable that the formulations used were “non-stealth” liposomes, well known to be targeted to primarily macrophages, which in humans typically do not express CD1a, CD1b, or CD1c (19, 20). Nonetheless, the protective effects are encouraging, setting the stage for improved lipid Ag vaccines targeting CD1+ DCs.

Delivery of Ag to the desired APC is a fundamental principle for successful vaccination. Although CD1d is constitutively expressed on B cells and myeloid cells, the group 1 CD1 isoforms, including CD1b, show more restricted expression on DCs (19–21), providing a specific cellular target for vaccine delivery. A major challenge for cell type–specific therapies such as targeted vaccines is to identify a receptor that is specific for the targeted cell, as well as a corresponding ligand or Ab that can be incorporated into the targeting platform. In the present study we used Siglec-7 as the targeted receptor owing to its known restricted expression on human myeloid cells. A recent study has shown that ~75% of Siglec-7+ myeloid cells in human gut lamina propria express CD11c, a DC marker, indicating the presence of Siglec-7+ DCs in human tissue (31). Furthermore, Lundberg et al. (39) have shown that human tonsillar DCs and blood DCs express the mRNA transcript of Siglec-7. Consistent with this, we found that human peripheral blood myeloid DCs, but not plasmacytoid DCs, express Siglec-7 protein (Supplemental Fig. 3). Several studies also show constitutive expression of Siglec-7 on monocytes and DCs during differentiation and maturation in vitro (32, 40). These data support the potential for use of Siglec-7–targeted liposomes for delivery of vaccines to DCs.

Although we have successfully targeted Siglec-7 expressed on DCs in this study, other siglecs may also represent attractive targets for human DC targeting. Transcript expression analysis suggests that Siglec-10 is highly expressed on various human DCs in skin, tonsil, and blood (39). Siglec-3, -5, and -9 have also been shown to be expressed on Mo-DCs (32), and Siglec-1 is induced on Mo-DCs in response to IFN-α (26). Given that both glycancoid and AB-based targeting technologies for siglecs are being actively developed (28, 29), verification of the in situ expression of these receptors in APCs of relevant human tissues will be needed to select the appropriate siglec to target.

For targeting DCs, we have employed a pegylated liposomal platform, wherein liposomes are decorated with glycan ligands of Siglec-7, and have established that these lead to robust delivery of mycobacterial lipid Ags to DCs. Pegylation is a well-established technique for increasing targeting specificity and avoiding nonspecific uptake by phagocytes (24). As a result, pegylated liposomes without the targeting glycancoid ligand (naked liposomes) were invisible to DCs and other Siglec-7+ leukocytes (Fig. 1) (34), leading to exquisite DC targeting specificity of the Siglec-7–targeted liposomes.

Key to this strategy is the fact that, similar to most other siglecs, Siglec-7 is an endocytic receptor, capable of efficiently internalizing targeted antigenic liposomes. The partial colocalization of Siglec-7–targeted liposomes with early endosomes and lysosomes is consistent with previous reports showing that poly(lactide-co-glycolide) nanoparticles decorated with an anti–Siglec-7 Ab are internalized and delivered to acidic endocytic compartments (33). The fact that colocalization is observed in both early endosomal and lysosomal compartments is consistent with a stepwise endocytic mechanism wherein endosomes precede, and eventually fuse with, lysosomes.

Delivery of lipid Ags to Mo-DCs results in robust activation of CD1b-restricted T cells relative to Mo-DCs exposed to free Ag or Ag in nontargeted liposomes. Furthermore, human myeloid DCs also express CD1a, CD1c, and CD1d, so the proof of principle for lectin targeting shown in the present study has potential applications for other lipid Ags delivered in liposomes. Although cross-linking of Siglec-7 by immobilized anti–Siglec-7 Ab has been reported to stimulate secretion of inflammatory cytokines by monocytes (41), it is notable that activation of T cells is Ag-dependent, because there is no activation with Siglec-7–targeted liposomes formulated without C80 GMM (Supplemental Fig. 4), excluding bystander effects resulting from induction of inflammatory cytokines. Thus, we conclude that Siglec-7 is a promising target for selective delivery of mycobacterial lipid Ags to DCs for improved vaccines against tuberculosis.

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Disclosures
The authors have no financial conflicts of interest.

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