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In situ Delivery of Antigen to DC-SIGN$^+$CD14$^+$ Dermal Dendritic Cells Results in Enhanced CD8$^+$ T-Cell Responses

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CD14$^+$ dendritic cells (DCs) present in the dermis of human skin represent a large subset of dermal DCs (dDCs) that are considered macrophage-like cells with poor antigen (cross)-presenting capacity and limited migratory potential to the lymph nodes. CD14$^+$ dDC highly express DC-specific ICAM-3-grabbing non-integrin (DC-SIGN), a receptor containing potent endocytic capacity, facilitating intracellular routing of antigens to major histocompatibility complex I and II (MHC-I andII) loading compartments for the presentation to antigen-specific CD8$^+$ and CD4$^+$ T cells. Here we show using a human skin explant model that the *in situ* targeting of antigens to DC-SIGN using glycan-modified liposomes enhances the antigen-presenting capacity of CD14$^+$ dDCs. Intradermal vaccination of liposomes modified with the DC-SIGN-targeting glycan Lewis^X, containing melanoma antigens (MART-1 or Gp100), accumulated in CD14$^+$ dDCs and resulted in enhanced Gp100- or MART-1-specific CD8$^+$ T-cell responses. Simultaneous intradermal injection of the cytokines GM-CSF and IL-4 as adjuvant enhanced the migration of the skin DCs and increased the expression of DC-SIGN on the CD14$^+$ and CD1a$^+$ dDCs. These data demonstrate that human CD14$^+$ dDCs exhibit potent cross-presenting capacity when targeted *in situ* through DC-SIGN.

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INTRODUCTION

Dendritic cells (DCs) have the ability to capture, process, and present antigens within the context of major histocompatibility complex I and II (MHC-I and-II) to CD8$^+$ and CD4$^+$ T lymphocytes, respectively, thereby initiating and maintaining adaptive immune responses (Steinman and Banchereau, 2007). DCs can present exogenous-derived antigens on MHC-I in a process known as cross-presentation. This phenomenon holds great interest, as it can directly contribute to the induction of antitumor CD8$^+$ T-cell responses.

Preferred strategies for DC targeting as cancer immunotherapy are based on the delivery of tumor antigens to DCs *in vivo*. This requires modification of antigens to allow recognition by specific DCs resulting in antigen internalization. Promising targets are the C-type lectin receptors (CLRs), which are expressed by distinct DCs and are known to internalize the antigen and to induce T-cell responses, such as DEC-205 (Hawiger *et al.*, 2001; Bonifaz *et al.*, 2002; Boscardin *et al.*, 2006) and DC-specific ICAM-3-grabbing non-integrin (DC-SIGN) (Singh *et al.*, 2009; Unger *et al.*, 2012). Most studies use monoclonal antibodies to target DC subsets (Hawiger *et al.*, 2001; Bonifaz *et al.*, 2002; Sancho *et al.*, 2008; Idoyaga *et al.*, 2011; Schreibelt *et al.*, 2012). However, antibodies can induce adverse immunogenic effects that obstruct the induction of successful antitumor responses (Chari, 2008). Using natural glycan ligands for CLRs would be a more versatile approach, as glycans are expressed throughout the body and therefore they are poorly immunogenic. DEC-205 has shown to be a potent lectin to internalize antigens and to induce antigen-specific T-cell responses after antigen targeting using DEC-205-specific antibodies (Idoyaga *et al.*, 2011). However, still no natural glycan ligands are described to bind to DEC-205 (Shrimpton *et al.*, 2009). Therefore, we excluded DEC-205 from analyses in this study.

It has been demonstrated that the modification of antigens using DC-SIGN-binding glycans resulted in efficient antigen internalization and increased T-cell responses

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Abbreviations: CLR, C-type lectin receptor; DC, dendritic cell; dDC, dermal dendritic cell; DC-SIGN, DC-specific ICAM-3-grabbing non-integrin; GP100, glycoprotein 100; Le^X, Lewis^X; mAbs, monoclonal antibody; MART-1, melanoma antigen recognized by T cell-1; MoDC, monocyte-derived dendritic cell; TLR, Toll-like receptor

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(Wang *et al.*, 2007; Aarnoudse *et al.*, 2008; Singh *et al.*, 2009; Unger *et al.*, 2012), making DC-SIGN an attractive receptor for targeting. Ligands for DC-SIGN comprise high-mannose oligosaccharides and Lewis-type epitopes, such as Lewis^X (Le^X) (Appelmelk *et al.*, 2003). DC-SIGN is highly expressed on *in vitro*–generated monocyte-derived DCs (moDCs), on DCs at mucosal sites, as well as in skin and lymph nodes (Engering *et al.*, 2004). The main populations of DCs that can be found in the human skin are the CD1a^{high}Langerin$^+$ Langerhans cells (LCs) present in the epidermis, the CD1a$^+$/CD1c$^+$ DC subset present in the dermis, and the CD14$^+$ dermal DCs (dDCs) (Klechevsky *et al.*, 2008; Klechevsky *et al.*, 2009; Segura *et al.*, 2012). In humans, DC-SIGN is primarily expressed by CD14$^+$ dDCs. LCs were shown to efficiently prime CD8$^+$ T cells, whereas CD14$^+$ dDCs induced the generation of follicular T-helper cells (Klechevsky *et al.*, 2008). CD1c$^+$ dDCs have recently been described as the functional equivalents of mouse CD11b$^+$ DCs and possess Th17-polarizing capacities (Schlitzer *et al.*, 2013). In addition, it has been shown that human CD1a$^+$ dDCs stimulated CD4$^+$ T-cell proliferation and primed CD8$^+$ T cells (Santegoets *et al.*, 2008). Whether *in situ* DC-SIGN targeting influences the T-cell priming capacities of the skin DC subsets is currently not known.

Here, we used glycan-modified liposomes to target DC-SIGN$^+$ DCs intradermally to induce tumor-specific T-cell responses. Liposomes are spherical nanoparticles, which can encapsulate large quantities of molecules, such as anti-tumor peptides. The human skin model resembles the physiological *in vivo* situation, allowing the examination of targeting specificity of vaccine formulation to DC subsets within the skin tissue, as well as the potential to alter the cross-presenting capacity of skin DC subsets to induce tumor-specific T-cell responses.

RESULTS

Le^X- and αDC-SIGN-modified liposomes are internalized by DC-SIGN

As previously reported, Le^X can bind with high affinity to DC-SIGN (Appelmelk *et al.*, 2003). We conjugated Le^X to liposomes to target DC-SIGN$^+$ DCs. As a positive control, we also modified liposomes with anti-DC-SIGN antibodies (αDC-SIGN). Indeed, DC-SIGN-Fc constructs bound to the Le^X and αDC-SIGN liposomes coated to ELISA plates (Figure 1a). Modification of liposomes with Le^X or αDC-SIGN resulted in enhanced binding to DC-SIGN and an increased internalization of modified liposomes by monocyte-derived dendritic cells (moDCs), as measured by an increase in the mean fluorescent intensity (MFI) (Figure 1b and Supplementary Figure S2 online). When cultured at 37 °C, all liposomes are internalized and are located inside the DCs (Supplementary Figure S2 online). Both the percentage of DiD$^+$ DCs and the MFI increased upon modification of the liposomes with Le^X glycans or αDC-SIGN antibodies (Supplementary Figure S1 online). Le^X-and αDC-SIGN-modified liposomes were equally well internalized by DC-SIGN$^+$ moDCs, indicating that glycans are as efficient to target DC-SIGN as antibodies. Besides DC-SIGN, moDCs do express other CLRs, such as the mannose receptor, Dendritic Cell ImmunoReceptor, and Macrophage Galactose-type Lectin (MGL) (Figure 1c). To exclude that these CLRs contributed to the liposomal internalization, we incubated moDCs with Le^X-modified liposomes in the presence of neutralizing mAbs against mannose receptor, MGL, DCIR, and langerin. As shown in Figure 1d, internalization of Le^X- and αDC-SIGN-modified liposomes was solely mediated through DC-SIGN, as the addition of neutralizing antibodies directed against the other CLRs did not hamper liposomal internalization. Neutralizing antibodies against DC-SIGN completely abrogated the internalization of Le^x-modified liposomes (Figure 1d).

DC-SIGN is mainly expressed by CD14$^+$ dDCs

We analyzed DC-SIGN expression on the three main migratory DC subsets present in the human skin. As shown in Figure 2, DC-SIGN is mainly expressed by the CD14$^+$ dDCs. Moderate expression of DC-SIGN was found on the CD1a$^+$ dDCs, whereas human LCs do not express DC-SIGN (Figure 2).

Intradermal injection of GM-CSF and IL-4 mobilizes and matures skin DCs

We investigated the potential of intradermally injected Toll-like receptor (TLR) ligands to mobilize and mature skin DCs, as these characteristics influence T-cell activation. We tested a panel of TLR ligands (the TLR3 ligand pI:C; the TLR4 ligand LPS and the TLR7/8 ligand R848) in the skin and analyzed the subset distribution and maturation state of emigrated CD14$^+$ and CD1a$^+$ dDCs. We also injected GM-CSF and IL-4 (GM/4), as administration of GM/4 has been shown to enhance migration of the phenotypically more mature CD1a$^+$ dDCs (de Gruijl *et al.*, 2006; Fehres *et al.*, 2014). Indeed, injection of GM/4 resulted in higher levels of migrated HLA-DR$^+$ DCs as compared with injection of medium or the TLR ligands (Figure 3a). Furthermore, GM/4 significantly reduced the percentage of migrated CD14$^+$ DCs from 33 to 8%, whereas the percentage of migrated CD1a$^+$ dDCs increased from 43 to 75% (Figure 3b). The TLR ligands did not affect the ratio of CD14$^+$/CD1a$^+$ dDCs. Although GM/4 reduced the percentage of CD14$^+$ dDCs, absolute numbers of migrated CD14$^+$ dDCs were unaffected by GM/4 administration compared with medium (Figure 3c, right panel). Rather, the decreased percentage of CD14$^+$ dDCs was caused by an increase in the absolute numbers of migrated CD1a$^+$ dDCs (Figure 3c, left panel).

Besides, intradermal injection of GM/4 increased the expression of DC-SIGN by CD14$^+$ and CD1a$^+$ dDCs, which was not observed upon injection of TLR ligands (Figure 3d and e). GM-CSF, in the absence of IL-4, was found to be the main factor responsible for the upregulation of DC-SIGN during *in vitro* moDC generation (Conti *et al.*, 2008). To determine whether the upregulation of DC-SIGN on skin DCs was induced by GM-CSF and/or IL-4, we administered IL-4, GM-CSF, or the combination intradermally and analyzed DC-SIGN expression. GM-CSF alone did increase DC-SIGN expression on both CD14$^+$ and CD1a$^+$

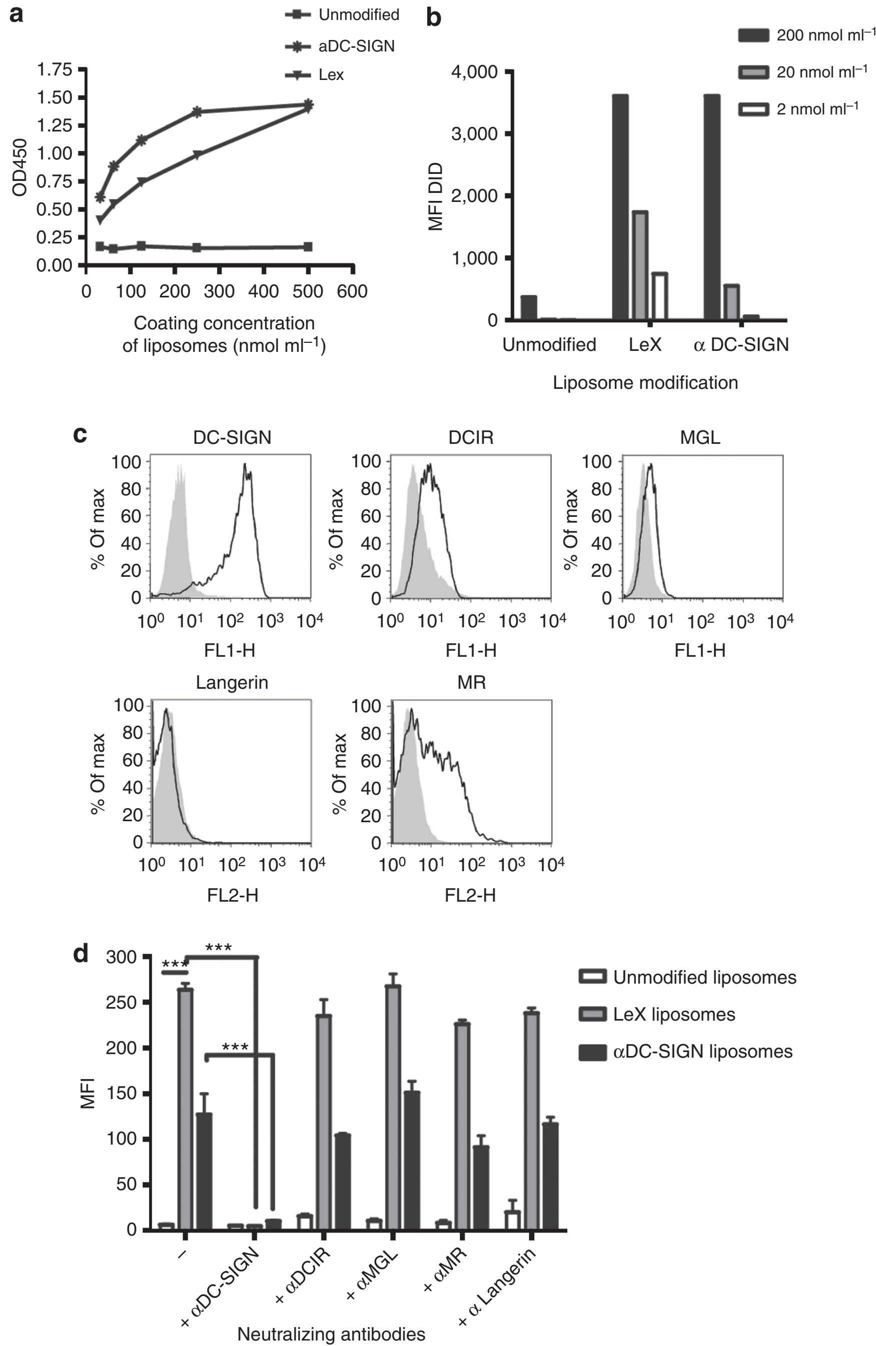

Figure 1. DC-specific ICAM-3-grabbing non-integrin (DC-SIGN) specifically binds to and internalizes Lewis^{X}- and αDC-SIGN-modified liposomes. (**a**) DC-SIGN binding to modified liposomes was tested using DC-SIGN-Fc molecules. Liposomes were coated to Nunc 96-well plates at indicated concentrations; $N=4$. (**b**) Internalization of DiD-labeled liposomes by DC-SIGN+ monocyte-derived dendritic cell (moDCs) measured by flow cytometry. Data shown depict a representative experiment; $N=4$. (**c**) Staining for C-type lectin receptors (CLRs) expressed by moDCs. Data from one representative experiment are shown; $N=2$. (**d**) Internalization of modified liposomes (20 nmol ml^{-1}) by moDCs is measured in the presence of indicated neutralizing antibodies against CLRs; $N=3$, mean ± SEM, $^{***}P<0.001$.

dDCs (Figure 3f). Again, CD14+ dDCs expressed higher levels of DC-SIGN, as indicated by a higher MFI compared with CD1a+ dDCs. Thus, intradermal vaccination of GM/4 increased the mobilization of dDCs and upregulated the expression of DC-SIGN on CD14+ dDCs and to some extent of CD1a+ dDCs.

Le^{X}- and αDC-SIGN-modified liposomes target specifically to CD14+DC-SIGN+ dDCs

Next, we investigated whether Le^{X}-modified liposomes are targeted to, and internalized by, DC-SIGN+ dDCs *in situ*. Two days after injection of liposomes with GM/4, emigrated dDCs were harvested, stained for HLA-DR, and liposome internali-

zation was studied. As we could not determine any liposomes outside of the DCs after incubation at 37 °C (Supplementary Figure S2 online) and the liposomes are partly degraded during the two-day migration of the dDCs after targeting, we did not investigate the crawl-out DCs using fluorescent microscopy, but analyzed the cells using flow cytometry. Modification of liposomes with Le^X or αDC-SIGN significantly enhanced liposome internalization by emigrated skin DCs (Figure 4a). Moreover, co-injection of neutralizing antibodies against DC-SIGN abrogated the internalization of Le^X- and αDC-SIGN-modified liposomes by dDCs (Figure 4a), indicative of DC-SIGN-mediated internalization.

As high expression of DC-SIGN is particularly found on $CD14^+$ dDCs, we analyzed liposome internalization on the $CD14^+$ and $CD1a^+$ dDCs separately. Consistent with the DC-SIGN expression in human skin, highest internalization of DC-SIGN-targeting liposomes was detected in the $CD14^+$ dDCs (Figure 4b). In addition, 45 and 68% of the $CD14^+$ dDCs had taken up Le^X- or αDC-SIGN-modified liposomes, respectively, compared with 28 and 51% of the $CD1a^+$ dDCs (Supplementary Figure S3A online). Although the percentages of $liposome^+$ cells were highest in the $CD14^+$ dDC subset, the main difference between the two dDC subsets was observed in the amount of liposomes taken up (measured by the MFI of

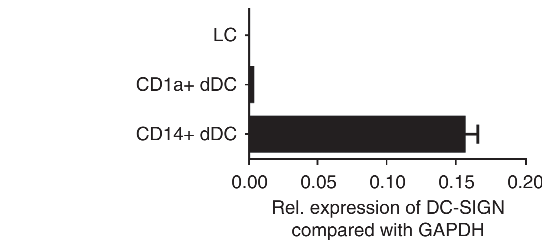

Figure 2. DC-specific ICAM-3-grabbing non-integrin (DC-SIGN) is mainly expressed by human $CD14^+$ dermal dendritic cells (DCs). Expression of DC-SIGN was analyzed on highly purified, FACS-sorted DC subsets present in the human skin using real-time PCR analyses; $N=3$, mean ± SEM is depicted. To ensure sufficiently high cell numbers, cells from at least four individual skin donors were pooled for each DC subset.

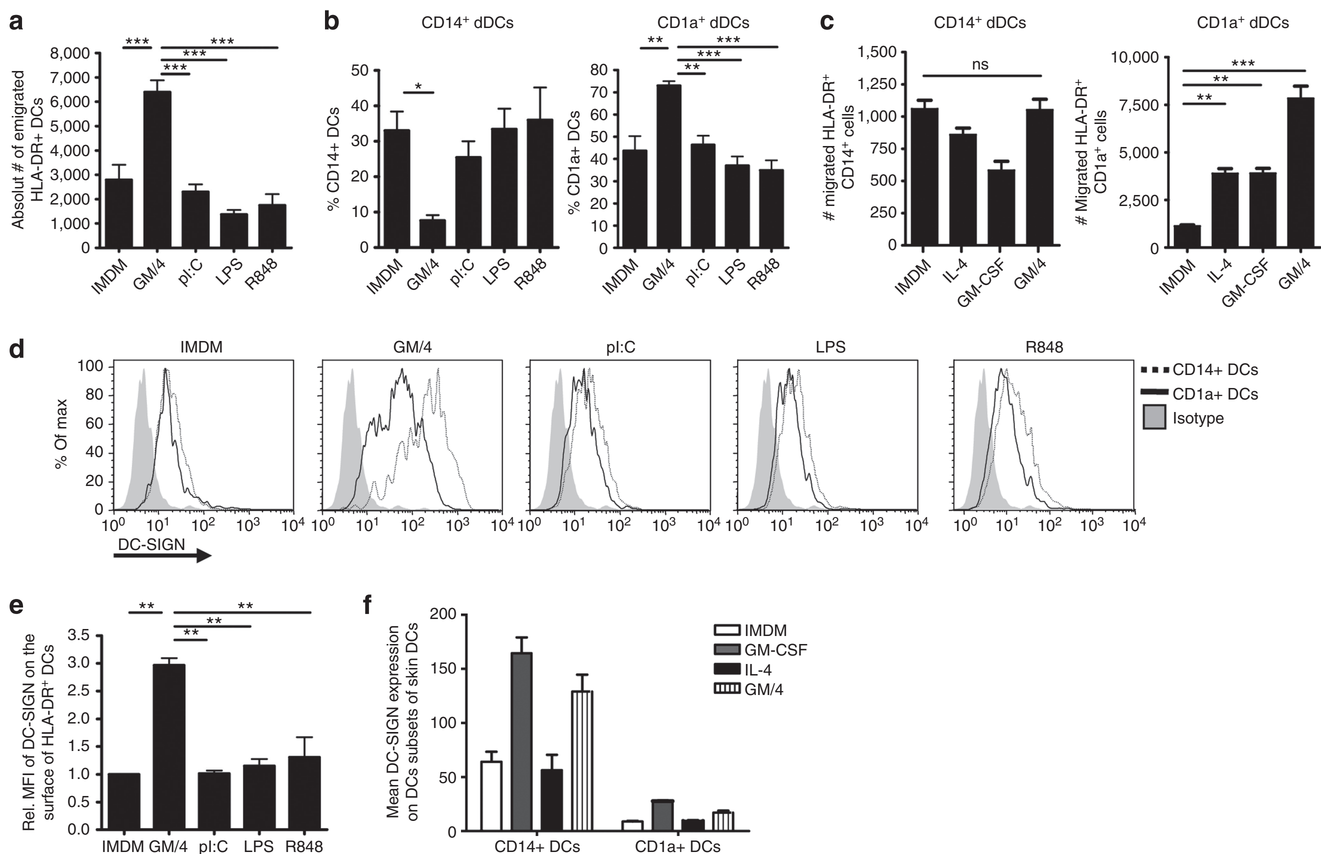

Figure 3. Intradermal injection of GM-CSF and IL-4 alters the subset balance of emigrated dendritic cells (DCs) and increases the expression of DC-specific ICAM-3-grabbing non-integrin (DC-SIGN). IMDM, GM/4, pI:C, LPS, or R848 were intradermally injected and numbers (**a**) and percentages (**b**) of migrated DCs were analyzed using flow cytometry. (**a**) Cells were stained for HLA-DR and measured during 1 minute of acquisition; $N=4$, means ± SEM, $^{***}P<0.001$. (**b**) CD1a and CD14 subset distribution was analyzed on emigrated $HLA\text{-}DR^+$ DCs; $N=4$, means ± SEM, $^{*}P<0.05$, $^{**}P<0.01$ or $^{***}P<0.001$. (**c**) Absolute numbers of $CD1a^+$ and $CD14^+$ dermal DCs (dDCs) were measured during 120 seconds of acquisition; $N=2$, means ± SEM, $^{**}P<0.01$ or $^{***}P<0.001$. (**d**) DC-SIGN expression on migrated $CD14^+$ and $CD1a^+$ dDCs after intradermal injection of indicated compounds. Cells were treated with saponin to measure total DC-SIGN levels (intracellular and surface expression). Results from one representative donor are shown; $N=3$. (**e**) Surface expression of DC-SIGN, depicted as relative increase compared with medium, was analyzed on $HLA\text{-}DR^+$ dDCs; $N=3$, mean ± SEM, $^{**}P<0.01$. (**f**) Surface DC-SIGN expression on migrated $HLA\text{-}DR^+$ $CD14^+$ and $CD1a^+$ dDCs; $N=3$, mean ± SEM.

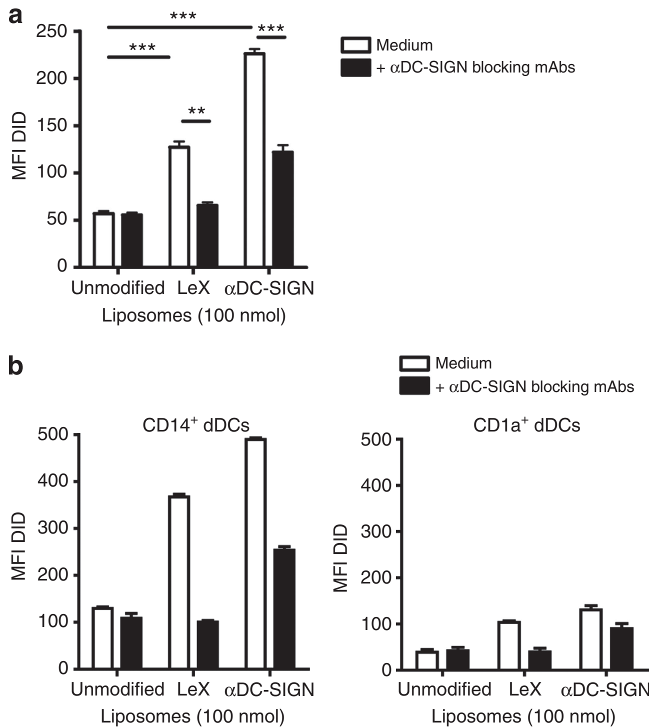

Figure 4. Lewis^X (Le^X)- and αDC-specific ICAM-3-grabbing non-integrin (DC-SIGN) -modified liposomes target CD14$^+$ dermal DCs (dDCs) and are internalized in a DC-SIGN-specific manner. (**a**) Simultaneous injection of DC-SIGN-blocking antibodies with Le^X- and αDC-SIGN-modified liposomes abrogated liposomal uptake by emigrated skin dendritic cells (DCs), as measured by flow cytometry on HLA-DR$^+$ DCs after 2 days of migration; $N=3$, means ± SEM. $^{**}P<0.01$, $^{***}P<0.001$. (**b**) Internalization of the liposomes by HLA-DR$^+$CD14$^+$ (left graph) or HLA-DR$^+$CD1a$^+$ (right graph) dDCs was measured using flow cytometry after 2 days of migration. $N=3$; results of one representative experiment are shown.

1,1′-Dioctadecyl-3,3,3′, 3′-Tetramethylindodicarbocyanine (DiD)). The MFI measured on the C14$^+$ dDCs after internalization of Le^X- or αDC-SIGN-modified liposomes was 363 and 487, respectively, whereas the MFI measured on the CD1a$^+$ dDCs after internalization of Le^X- or αDC-SIGN-modified liposomes was 101 and 124, respectively (Figure 4B). Again, neutralizing antibodies against DC-SIGN decreased the internalization of modified liposomes (Figure 4b).

In situ skin targeting of DC-SIGN using Le^X-modified liposomes enhances antigen presentation to CD8$^+$ T cells by CD14$^+$ dDCs

Liposomal internalization via DC-SIGN may facilitate antigen presentation to CD8$^+$ T cells, as DC-SIGN can route antigen to MHC-I loading compartments (Unger *et al.*, 2012; Garcia-Vallejo *et al.*, 2013). Therefore, Le^X-modified liposomes containing a 15-aa-long melanoma antigen recognized by T cells-1 (MART-1) peptide or a 9-aa-long Gp100 peptide were generated and injected intradermally in the presence of GM/4. As we did not observe a difference between the Le^X- and αDC-SIGN-modified liposomes in the antigen-presentation assays after internalization by moDCs (Supplementary Figure S4 online), we focused on the comparison between unmodified and Le^X-modified liposomes in experiments using human skin. Migratory DCs were tested in antigen-presentation assays using a CD8$^+$ T-cell clone specific for MART-1$_{26\text{-}35(27L)}$ or Gp100$_{280\text{-}288}$. Indeed, enhanced dose-dependent antigen presentation of the MART-1 peptide to the CD8$^+$ T cells was observed when the liposomes were coated with Le^X and targeted to DC-SIGN$^+$ dDCs, as measured by significantly increased production of IFN-γ (Figure 5a).

As we observed the highest internalization of the liposomes in the CD14$^+$ dDCs (Figure 4b) and this subset has been described to lack the potential to cross-present antigens (Klechevsky *et al.*, 2008; McGovern *et al.*, 2014), we determined which dermal DC subpopulation contributed to the observed effects on antigen presentation. Using magnetic-activated cell sorting (MACS) isolation, we separated migrated CD14$^+$ dDCs from the CD1a$^+$ dDCs and incubated the DC subsets with Gp100 peptide–containing liposomes and GM/4, after which dDCs were cocultured with Gp100-specific CD8$^+$ T cells. MACS isolation resulted in an enrichment of separated CD1a$^+$ and CD14$^+$ dDCs (Supplementary Figure S5 online). We also analyzed CCR7 expression on the purified subsets (Supplementary Figure S6 online). Highest expression of CCR7 was found on the CD1a$^+$ dDCs, which is in line with previously reported data (McGovern *et al.*, 2014). However, the CD14$^+$ dDCs did also express CCR7. With regard to CD8$^+$ T-cell activation, Gp100-specific CD8$^+$ T-cell reactivity was enhanced upon coculture with DC-SIGN-targeted CD14$^+$ dDCs but not with similarly targeted CD1a$^+$ dDCs, providing evidence that primarily the CD14$^+$ dDCs are capable of inducing CD8$^+$ T-cell activation after antigen internalization through DC-SIGN (Figure 5b). To exclude the possibility of an intrinsic difference in antigen-presenting capacity between the DC subsets, we loaded the CD14$^+$ and CD1a$^+$ dDCs with the 9-aa-long Gp100 peptide, which can directly bind the MHC-I, and co-cultured the pulsed cells with the Gp100-specific T-cell clone. We could not observe a difference between the two subsets in their capacity to activate the CD8$^+$ T cells, showing that both dDC subsets were equally potent to present antigens in MHC-I (Supplementary Figure S7 online). Consequently, the differences in the capacity to induce CD8$^+$ T-cell responses between the CD14$^+$ and CD1a$^+$ dDCs after liposomal internalization are likely caused by the targeting through DC-SIGN.

DISCUSSION

Here we demonstrate that Le^X-modified liposomes target antigens to DC-SIGN-expressing DCs within the human skin. Moreover, simultaneous administration of GM/4 enhanced the DC-SIGN expression, facilitating a DC-SIGN-mediated internalization of Le^X-modified liposomes preferentially by CD14$^+$ dDCs and subsequent antigen presentation to, and activation of, CD8$^+$ T cells.

Using the human skin explant model, we were able to assess the internalization and fate of antigen-encapsulated liposomes *in situ*. This model simulates the migration of DCs toward the draining lymph nodes, which is the location at

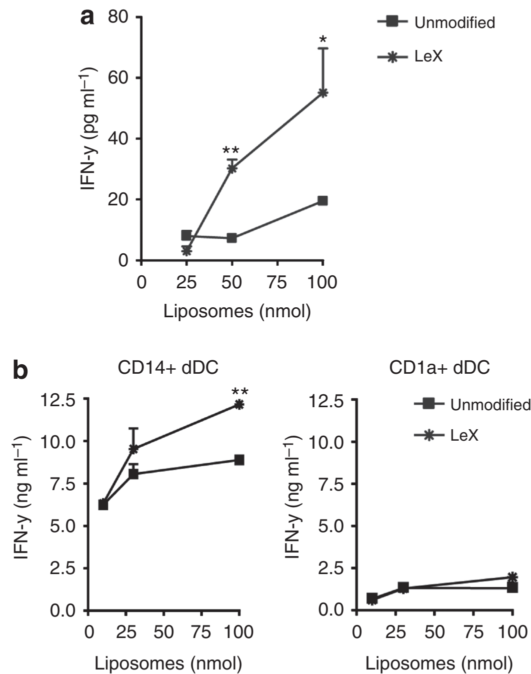

Figure 5. Intradermal targeting of Lewis^X (Le^X)-modified liposomes results in enhanced CD8$^+$ T-cell reactivity. (**a**) HLA-A2$^+$ skin was injected intradermally with melanoma antigen recognized by T cells-1 (MART-1)-containing liposomes resuspended in medium + GM/4. Emigrated DCs were harvested after 2 days and cocultured with an HLA-A2-restricted, MART-1-specific CD8$^+$ T clone at a ratio of 1:5. After 24 hours, IFN-γ was measured using ELISA; $n=3$, mean ± SEM, $*P<0.05$ and $**P<0.01$, using a two-way analysis of variance (ANOVA). (**b**) MACS-sorted CD14$^+$ and CD1a$^+$ dDCs were allowed to internalize GP100-containing liposomes and cocultured with an HLA-A2-resricted, GP100-specific CD8$^+$ T clone at a ratio of 1:5. After 24 hours, IFN-γ was analyzed using ELISA; $N=3$, mean ± SEM, $**P<0.01$ as measured by a two-way ANOVA.

which naive antigen-specific T cells will be primed and activated. Lymph node migration is directed by CCL19 and CCL21 signaling through CCR7. Recently, it was shown that CD14$^+$ dDCs did not express CCR7, even upon stimulation with LPS, IL-1β, and TNF-α. However, spontaneous migration of the CD14$^+$ dDCs from the skin was observed, suggesting that CD14$^+$ migratory dDCs exited the skin without entering the lymphatics (McGovern *et al.*, 2014). Here, we have demonstrated that CD14$^+$ dDCs do also express CCR7 after spontaneous migration, indicating that these cells might migrate to draining lymph nodes. Definitive prove showing the capacity of CD14+ dDCs to migrate to the lymph nodes cannot be tested in the human skin explant model and is a topic that still needs to addressed. In addition, antigen-loaded and matured skin-resident dDCs could also add to the antitumor responses induced by the vaccine by providing local antigen-specific signals that stimulate effector T cells, especially at an immunosuppressive tumor site.

Both Le^X- and the αDC-SIGN-modified liposomes were taken up by the DC-SIGN$^+$ dDCs to a significantly higher extent as compared with nontargeted liposomes. In addition, similar CD8$^+$ T-cell responses were observed when moDCs were targeted with Le^X- or αDC-SIGN-modified liposomes. The use of natural ligands to target DC-SIGN *in vivo* is preferred over the use of DC-SIGN-specific antibodies, as even humanized antibodies might induce adverse immunogenic effects that obstruct the induction of anti-tumor responses.

Within the human skin, DC-SIGN is predominantly expressed by the CD14$^+$ dDCs. We have shown that the targeting of DC-SIGN$^+$ dDCs under inflammatory conditions using GM/4 resulted in significantly enhanced antigen cross-presentation to CD8$^+$ T cells primarily by the CD14$^+$ dDCs. In particular, the activation of antigen-specific CD8$^+$ T cells by the CD14$^+$ dDCs after DC-SIGN-mediated internalization of antigen is an important finding, as those cells have previously been described as poor activators of CD8$^+$ T cells (Klechevsky *et al.*, 2008; Haniffa *et al.*, 2012; Garcia-Vallejo *et al.*, 2013). However, we clearly show here that MACS-sorted CD14$^+$ dDCs induced higher activation of antigen-specific effector CD8$^+$ T cells than CD1a$^+$ dDCs, providing evidence that CD14$^+$ dDCs can present antigens efficiently after DC-SIGN-mediated internalization.

The apparent discrepancy of our observation concerning the potential of CD14$^+$ dDCs to induce CD8$^+$ T-cell responses to those of others may be dependent on the method to induce these responses. In our study, we have analyzed the capacity of DCs to present antigens that were specifically internalized via DC-SIGN. Others have contributed the weak CD8$^+$ T-cell stimulatory potential of the CD14$^+$ dDCs based on their capacity to cross-present soluble peptides, apoptotic cells, or untargeted antigens (Haniffa *et al.*, 2012; Segura *et al.*, 2012). It is possible that the DC-SIGN-mediated uptake of antigen favors efficient antigen routing to MHC-I processing and loading compartments. DC-SIGN targeting thereby overrules the poor cross-presenting potential of untargeted antigens by CD14$^+$ dDCs by affecting the intracellular antigen routing. This is supported by data of our own group and others (Tacken *et al.*, 2005; Singh *et al.*, 2009; Unger *et al.*, 2012), in which targeting of antigen to human DC-SIGN was ~100-fold more efficient in inducing T-cell responses than soluble antigens.

Another possible explanation may be the intradermal injection of GM/4. We show that GM/4 resulted in an increased expression of DC-SIGN on both the migrated CD1a$^+$ and CD14$^+$ dDC subsets, although the levels were lower on the CD1a$^+$ dDCs (Figure 3f). It is currently unknown whether GM/4 can induce higher expression of DC-SIGN on DC subsets already positive for DC-SIGN or whether it may induce *de novo* expression on cells present in the intact skin microenvironment. From *in vitro* studies using blood monocytes, it is known that GM-CSF and IL-4 induce the differentiation from DC-SIGN^{neg}CD14$^+$ monocytes to CD1a$^+$ moDCs with a high expression of DC-SIGN (Seager *et al.*, 2004). Intradermal administration of GM/4 to the skin not only alters the expression of DC-SIGN, it also resulted in the increased migration of the phenotypically more mature CD1a$^+$ dDCs (de Gruijl *et al.*, 2006).

The specific targeting of DC-SIGN$^+$ dDCs using glycosylated liposomes eliminated the BDCA3$^+$ dDC subset from our analyses, as it has been described that these cells do not

express DC-SIGN (Chu *et al.*, 2012). Although the $CD14^-BDCA3^+$ DCs has been described as superior antigen cross-presenting cells, they represent a minor population in the skin, which makes them less suitable for targeted immunotherapies (Haniffa *et al.*, 2012). In addition, antigen delivery to early endosomes through internalization via CD40 or CD11c eliminated the superior capacity of human blood $BDCA3^+$ DCs, resulting in a similar efficiency at cross-presentation as the $BDCA1^+$ DCs, which demonstrates the importance of the route of antigen internalization with regard to cross-presentation (Cohn *et al.*, 2013). The results presented here are in line with these findings, as we have demonstrated that antigen internalization through DC-SIGN allowed the $CD14^+$ dDCs to present antigens in MHC-I and to activate $CD8^+$ T cells.

In conclusion, conjugation of Le^X or αDC-SIGN antibodies to liposomes facilitated *in situ* targeting of $DC\text{-}SIGN^+$ DCs after intradermal vaccination, resulting in the efficient delivery of the liposomal cargo to $DC\text{-}SIGN^+$ DCs. Furthermore, DC-SIGN-mediated internalization of Le^X-modified liposomes resulted in enhanced antigen presentation by GM/4-stimulated dDCs and subsequently increased antigen-specific $CD8^+$ T-cell responses, mainly through $CD14^+$ dDCs. Therefore, the combined administration of glycoliposomes with GM/4 as adjuvant represents an efficient system to specifically deliver antigens to DC subsets for the induction of CD8 T-cell responses, and it should be considered a promising strategy for the development of targeted antitumor immunotherapy.

MATERIALS AND METHODS

DC isolation from human skin explants

LCs and $CD14^+$ and $CD1a^+$ dDCs were isolated from human skin derived from abdominal resections from healthy donors (Bergman Clinics, Bilthoven, The Netherlands) within 24 hours after plastic surgery. Material was obtained with informed consent (information leaflet for use of 'left-over' material), which was approved by the Medical Ethical Committee of Vumc. Skin was incubated with dispase (Roche, Basel, Switzerland) for 16 hours at 4 °C, followed by the separation of epidermal and dermal sheets using tweezers. Epidermal sheets were cut in pieces and incubated for 30 min at 37 °C in PBS containing trypsin and DNase, after which the cells were run over a 100-μm cell strainer to obtain a single-cell suspension. LCs are purified using a Ficoll gradient, resulting in ~ 90% pure LCs. To isolate dDCs, dermal sheets were cut in small pieces and incubated in collagenase A, dispase, and DNase for 2 hours at 37 °C. A single-cell suspension was obtained by putting the suspension over a 100-μm cell strainer. Where indicated, migrated dDCs and LCs were MACS-sorted using CD1a and CD14 microbeads (MACS, Miltenyi Biotec, Leiden, The Netherlands) or DC subsets were FACS-sorted using a MoFlo cell sorter (Beckman Coulter, Woerden, The Netherlands) and fluorescent antibodies directed against HLA-DR, CD1a, and CD14.

Intradermal injection and culture of skin biopsies

Liposomes were diluted in serum-free medium (IMDM) and injected intradermally, as described previously (Fehres *et al.*, 2014). Biopsies were taken after injection using a 6-mm biopsy punch (Microtek, Zutphen, The Netherlands) and cultured in a 48-well plate containing 1 ml of IMDM supplemented with 10% FCS, 10 μg ml^{-1} gentamycin, penicillin, and L-glutamine for 48 hours, at 37 °C and 5% CO_2. In each experiment, 10–15 biopsies were taken per condition. After 48 hours of culture, the biopsies were discarded and emigrated DCs were harvested and used for experiments.

Phenotypic analysis of crawl-out cells

Analysis of emigrated cells was performed by flow cytometry, as previously described (Fehres *et al.*, 2014). Fluochrome-conjugated mAbs used were specific for CD1a, CD14, CD70, CD86, DC-SIGN, HLA-DR (BD, San Jose, CA), HLA-ABC (ImmunoTools, Friesoythe, Germany), CD83 (Beckman Coulter Immunotech, Prague, Czech Republic), CCR7 (R&D Systems), or isotype-matched control mAbs (BD). For the intracellular DC-SIGN staining, the cells were treated with 0.1% saponin for 30 minutes at RT, washed, and consequently stained with DC-SIGN, CD1a, and CD14.

Real-Time PCR

FACS-sorted $CD14^+$ dDCs, $CD1a^+$ dDCs, and LCs were pooled from at least four human skin donors to obtain high enough numbers for analysis. Pooled cells were lysed and mRNA was isolated using an mRNA Capture kit (Roche). cDNA was synthesized using the Reverse Transcription System kit (Promega, Madison, WI) according to the manufacturer's guidelines. Oligonucleotides were designed using the Primer Express 2.0 software (Life Technologies Europe, Bleiswijk, The Netherlands) and synthesized by Invitrogen Life Technologies (Invitrogen, Carlsbad, CA). Real-Time PCR analysis was performed as previously described using the SYBR Green method in an ABI 7900HT sequence detection system (Applied Biosystems) (van Vliet *et al.*, 2006). *GAPDH* was used as an endogenous reference gene.

Liposome preparation

Liposomes were prepared as previously described (Joshi *et al.*, 2011). The $MART\text{-}1_{21\text{-}35}$ or $Gp100_{280\text{-}288}$ peptides were encapsulated in the liposomes, as previously described (Unger *et al.*, 2012). Peptides were produced by solid-phase peptide synthesis using Fmoc double-coupling chemistry with a Symphony peptide synthesizer (Protein Tecnologies, Tucson, AZ).

$Lewis^X$ (Elicityl, Crolles, France) or anti-DC-SIGN antibody coupling to the liposomes was done using thiol-maleimide chemistry. To this end, a thiol group was incorporated to the glycans through derivatization of the glycans with cysteamine (Sigma-Aldrich, St Louis, MO) and the antibodies with *N*-succinimidyl S-acetylacetate (Thermo Scientific, Waltham, MA). In brief, lyophilized glycans were dissolved in dimethyl sulfoxide/acetic acid (8:2), and to this solution 10 equivalents (eq.) of cysteamine was added. After reacting at 65 °C for 20 minutes, 20 eq. of 2-picoline-borane (Sigma-Aldrich) was added and the mixture was stirred for 2 hours at 65 °C, followed by purification by normal-phase HPLC on a Zorbax-NH_2 prep column (Agilent Technologies, Santa Clara, CA; elution water/acetonitrile, gradient 85 to 15% of acetonitrile in 30 minutes). After lyophilization of the collected fractions, the resulting dry powder was dissolved in water and treated with 20 eq. of tris(2-carboxyethyl)phosphine (TCEP, Sigma-Aldrich). After 1 hour, the thio-glycan solution was purified using disposable sephadex G10 columns equilibrated with 50 mM ammonium formate (Sigma-Aldrich). Glycan derivatization was confirmed by ESI-MS (Thermo Finnigan LCQ-Deca XP Iontrap mass spectrometer in positive mode using a nanospray capillary needle). Antibodies were dissolved in hepes buffer, and 8 eq. of *N*-

succinimidyl S-acetylacetate dissolved in a minimum amount of dimethyl formamide was added. After 45 min at room temperature, the protein was washed three times over Vivaspin filters (10-kDa cutoff, Sartorius, Goettingen, Germany) and then the acetyl group of *N*-succinimidyl S-acetylacetate was removed by reaction with a 1:10 solution of hydroxylamine (Sigma-Aldrich) for 1 h. Subsequently, the yielded thio-glycans or thio-antibodies were coupled to the liposomes through a thiol-ene reaction with maleimide groups of the 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl) butyramide] lipid. 0.1% of the fluorescent lipophilic dye 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt (DiD; Invitrogen) was added to the liposome preparation to allow visualization after uptake by cells using flow cytometry.

Detection of glycans using ELISA

The conjugation of Le^X to the liposomes was confirmed by ELISA using anti-Le^X antibodies (Calbiochem, Damstadt, Germany), and correct orientation of the glycans was assessed using DC-SIGN-Fc molecules, as previously described (Unger *et al.*, 2012). Briefly, liposomes were coated onto NUNC maxisorb plates (Roskilde, Denmark) and incubated overnight at 4 °C. Plates were blocked with 1% BSA in PBS to avoid nonspecific binding. After extensive washing, the liposomes were incubated with anti-Le^X antibodies or DC-SIGN-Fc for 1.5 hours at RT. Binding was detected using a peroxidase-labeled F(ab′)2 goat anti-mouse IgG/Fcγ-specific antibody or an F(ab′)2 goat anti-human IgG/Fcγ-specific antibody, respectively. The reaction was developed and optical density was measured at 450 nm. As a positive control, Le^X attached to polyacrylamide (PAA) (Lectinity, Moscow, Russia) was used.

Liposome internalization by human moDCs

Human immature moDCs were generated and cultured, as previously described (Unger *et al.*, 2012). Liposomal uptake was analyzed by FACS following 3 hours of incubation at 37 °C. When indicated, $20\,\mu g\,ml^{-1}$ neutralizing antibodies against DC-SIGN (Geijtenbeek *et al.*, 2000), mannose receptor (clone 19.3, BD Biosciences), Macrophage Galactose-type Lectin (clone 125A10.03, Dendritics, Lyon, France), Dendritic Cell ImmunoReceptor (clone 111F8.o4, Dendritics), or langerin (de Witte *et al.*, 2007) was added.

Liposome internalization via DC-SIGN by human skin DC subsets

Liposomes were diluted in serum-free medium (IMDM) and injected intradermally, as described previously (Fehres *et al.*, 2014). When indicated, 0.4 μg of neutralizing αDC-SIGN antibody (AZN-D1) was co-injected per biopsy. Biopsies were taken after injection using a 6-mm biopsy punch (Microtek) and cultured in a 48-well plate containing 1 ml of IMDM supplemented with 10% FCS, $10\,\mu g\,ml^{-1}$ gentamycin, penicillin, and L-glutamine for 48 hours at 37 °C and 5% CO_2. After 48 hours of culture, the biopsies were discarded, emigrated DCs were harvested, and DC-SIGN-mediated internalization of DiD^+ liposomes was measured using flow cytometry.

Antigen presentation to human MART-1-specific or GP100-specific $CD8^+$ T-cell clone

An HLA-A2-restricted $CD8^+$ T-cell clone specific for MART-1_{26-35} was generated and cultured as described previously (Hooijberg *et al.*, 2000), as well as the GP100-specific $CD8^+$ T-cell clone (Schaft *et al.*, 2003). Indicated concentrations of liposomes resuspended in medium containing GM/4 were intradermally injected in the human skin in the presence or absence of $20\,\mu g\,ml^{-1}$ neutralizing antibody against DC-SIGN (AZN-D1). After 2 days, emigrated HLA-$A2^+$ skin cells were harvested and 2×10^4 cells/well were seeded in a 96-well round-bottom plate. After extensive washing, MART-1-specific or GP100-specific $CD8^+$ T cells (10^5/well) were added. After 24 hours, supernatants were taken and IFN-γ levels were measured by sandwich ELISA using specific antibody pairs from Biosource (San Diego, CA).

Statistical analysis

Results were analyzed using a one-way ANOVA followed by Bonferrroni Multiple Comparison test. When stated, the two-way ANOVA was performed using GraphPad Prism software (GraphPad Software, San Diego, CA). Results were considered to be significantly different when $P<0.05$, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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