# S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions

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**Dendritic cells (DCs) are antigen-presenting cells that play an essential role in mucosal tolerance. They regularly encounter beneficial intestinal bacteria, but the nature of these cellular contacts and the immune responses elicited by the bacteria are not entirely elucidated. Here, we examined the interactions of *Lactobacillus acidophilus* NCFM and its cell surface compounds with DCs. *L. acidophilus* NCFM attached to DCs and induced a concentration-dependent production of IL-10, and low IL-12p70. We further demonstrated that the bacterium binds to DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN), a DC- specific receptor. To identify the DC-SIGN ligand present on the bacterium, we took advantage of a generated array of *L. acidophilus* NCFM mutants. A knockout mutant of *L. acidophilus* NCFM lacking the surface (S) layer A protein (SlpA) was significantly reduced in binding to DC-SIGN. This mutant incurred a chromosomal inversion leading to dominant expression of a second S layer protein, SlpB. In the SlpB-dominant strain, the nature of the interaction of this bacterium with DCs changed dramatically. Higher concentrations of proinflammatory cytokines such as IL-12p70, TNF$\alpha$, and IL-1$\beta$ were produced by DCs interacting with the SlpB-dominant strain compared with the parent NCFM strain. Unlike the SlpA-knockout mutant, T cells primed with *L. acidophilus* NCFM stimulated DCs produced more IL-4. The SlpA–DC-SIGN interaction was further confirmed as purified SlpA protein ligated directly to the DC-SIGN. In conclusion, the major S layer protein, SlpA, of *L. acidophilus* NCFM is the first probiotic bacterial DC-SIGN ligand identified that is functionally involved in the modulation of DCs and T cells functions.**

lactobacilli | DC-SIGN

Over a long evolutionary period, lactobacilli have been abundant colonizers of the human small intestinal mucosa coexisting in mutualistic relationships with the host. Some members of the group exert additional probiotic properties providing health benefits to the host via regulation of immune system functions. Of these, *Lactobacillus acidophilus* NCFM is one of the most widely recognized and commercially distributed probiotic cultures (1, 2). Although cell surface components of *L. acidophilus* NCFM and other lactobacilli resident in the human gastrointestinal (GI) tract could activate the functions of various antigen-presenting cells, the mechanisms of such immune modulations are largely unknown. The detailed characterization of *L. acidophilus* NCFM components that are effectors of the immune system is, therefore, critical for understanding host–microbial interplays and modes of action of commensal and probiotic bacteria in the intestine.

Dendritic cells (DCs) are professional antigen-presenting cells that regularly interact with intestinal bacteria, including lactobacilli at various mucosal sites. DCs play an essential role in bridging innate and adaptive immunity integrating various exogenous and indigenous stimuli and initiating the appropriate immune responses or tolerance (3, 4). Precursors of immature dendritic cells (iDCs) migrate through the bloodstream and home to various tissues including several compartments of the human gut where they can interact with protruding pathogenic and nonpathogenic bacteria. DCs then undergo phenotypic and functional changes, such as up-regulation of cell surface expression of costimulatory and adhesion molecules and production of inflammatory chemokines and cytokines. Along with antigen uptake and processing, these functional changes in the DCs initiate both humoral and adaptive immune responses. Depending on the microbial stimulus encountered, DCs can promote the differentiation of unprimed, naïve T cells toward Th1, Th2, unpolarized T cells, or T regulatory cell responses (4, 5).

A variety of pattern recognition receptors (PRRs) are expressed on iDCs recognizing characteristic molecular patterns within microbial carbohydrates, lipids, nucleic acids, and proteins of protruding pathogens or abundant commensal bacteria (6). Such receptors include the Toll-like receptors (TLRs) (7) and the C-type lectins (CLRs) (8). TLRs relay information from the interacting microbial compounds to DCs through intracellular signaling cascades, thereby eliciting appropriate cellular processes, such as DCs maturation and/or the induction of proinflammatory cytokines (IL-12, IFN$\gamma$) (7). In contrast, CLRs recognize carbohydrate structures on self- and nonself-antigens followed by their processing and presentation, without induction of DC maturation (9). Thus far, >15 CLRs have been identified on DCs and macrophages (10). DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) is a CLR expressed mainly on DCs and recognizes mannose- and fucose-containing glycans that are present on endogenous and on microbial or viral surfaces. Because of these characteristics, DC-SIGN is implicated to play a role in the induction of various responses mediated by DCs. Several pathogens such as *Helicobacter pilori*, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Neisseria meningitides*, and *Schistosoma mansoni* could interact with DC-SIGN and modulate responses (11–15).

Recently, it was found that *Lactobacillus reuteri* and *Lactobacillus casei* can also bind to DC-SIGN and lead to the induction of regulatory T cells (4). The detailed molecular mechanisms by which beneficial bacteria, including lactobacilli, interact with DC-SIGN to modulate immune responses and promote mucosal homeostasis are not completely understood. In this work, we

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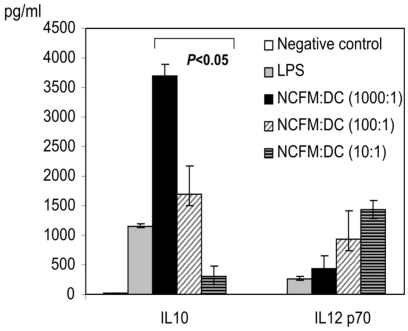


**Fig. 1.** *L. acidophilus* NCFM induces concentration-dependent iDC cytokine responses. iDCs were incubated with *L. acidophilus* NCFM, LPS (10 ng/mL), or no supplement for 2 days at 37 °C. DCs supernatants were harvested after 48 h and analyzed for IL-10 and IL-12p70. Experiments were repeated 3 times, and the values are the average ± SD.

investigated the molecular receptor/ligand interactions between the DCs and *L. acidophilus* NCFM and their contributions to the subsequent DC-driven Th1/Th2 differentiation.

## Results

**Cytokine Production by *L. acidophilus* NCFM-Treated DCs.** Previous studies have shown that DCs undergo maturation after interaction with lactobacilli, as measured by expression of cell surface markers and cytokines (5, 16, 17). To determine the levels of *L. acidophilus* NCFM attachment, bacteria were labeled with FITC and incubated at varying ratios, ranging from 1,000 to 10 cfu of *L. acidophilus* NCFM per 1 iDC, for 45min at 37 °C. Concentration-dependent bacterial binding was observed when the amount of the fluorescence detected by flow cytometry within the population of iDCs, was taken as a measure of the *L. acidophilus* NCFM adherence at the examined ratios [supporting information (SI) Fig. S1]. Furthermore, the effect of different concentrations of *L. acidophilus* NCFM on the expression of antiinflammatory (IL-10) and proinflammatory (IL-12p70) cytokines was assessed. The level of expression of these 2 cytokines was strongly influenced by the concentrations of *L. acidophilus* NCFM during the maturation of iDCs (Fig. 1) DCs incubated with the bacterium at ratio 1,000:1 (bacteria:iDCs) produced significantly higher IL-10 compared with a ratio of 10:1. In contrast, IL-12p70 was up-regulated at a lower concentration of the bacterium (10:1).

Next, the expression of HLA-DR and costimulatory molecules by *L. acidophilus* NCFM maturated DCs was examined. The bacteria modulated the iDC phenotype by up-regulating HLA-DR and activated the expression of the costimulatory molecules CD80 and CD86 at all examined ratios (1,000:1, 100:1 and 10:1; data not shown).

***L. acidophilus* NCFM Ligate DC-SIGN on Monocyte-Derived DCs via SlpA.** The role of particular PRRs in the bacterial–DC interactions was further evaluated. Specific C-type lectin receptors are highly expressed by DCs and bind carbohydrates on pathogens and commensals in a calcium-dependent manner. Ligation of *L. acidophilus* NCFM to iDCs was significantly reduced by the $Ca^{2+}$ chelator EGTA, suggesting a role for the C-type lectins PRRs (Fig. S2). Earlier studies indicated that DC-SIGN is a major C-type lectin receptor on iDCs recognizing *L. reuteri* and *L. casei* (4). When the binding of *L. acidophilus* NCFM to this receptor was examined by using ELISA, a strong ligation of the bacteria to the DC-SIGN-Fc was observed (Fig. 2). Blocking studies revealed that this bacterium specifically interacted with DC-SIGN because neutralizing anti-DC-SIGN Ab or EGTA blocked the binding completely. Furthermore, no binding to *L. acidophilus* NCFM was obtained after using a DCs immunoreceptor fused to Fc (DCIR-Fc) as a control for the Fc binding in the ELISA (Fig. 2). To investigate whether cellular DC-SIGN binds *L. acidophilus* NCFM, we used CHO cells expressing DC-SIGN. Binding of *L. acidophilus* NCFM was significantly higher to CHO cell line transfected with DC-SIGN compared with mock (CHO cells alone) (Fig. 3). After the addition of DC-SIGN-specific antibody and EGTA, the *L. acidophilus* NCFM ligation to CHO cell line expressing DC-SIGN was reduced to the levels of attachment to mock. Thus, DC-SIGN functions as a cellular receptor for *L. acidophilus* NCFM.

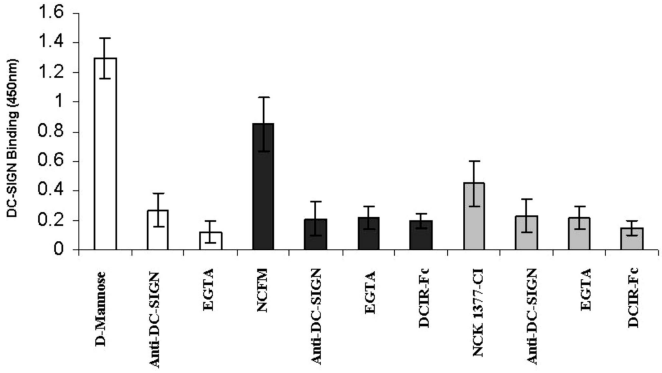


**Fig. 2.** Differential binding of DC-SIGN-Fc to *L. acidophilus* NCFM ($SlpA^+$) and NCK1377-CI ($SlpB^+$). By using the ELISA system, the DC-SIGN-Fc-binding specificity was determined in the presence of EGTA and blocking antibody to DC-SIGN (AZN-D1) and DCIR-Fc. *L. acidophilus* NCFM indicates *L. acidophilus* NCK1377-CI. All results are representative for 3 independent experiments (average ± SD); *, $P < 0.05$.

To identify the specific DC-SIGN ligand expressed on the *L. acidophilus* NCFM cell surface, we took advantage of a number of *L. acidophilus* NCFM-knockout mutants that were generated (Table S1 and ref. 18). When the various mutants were examined for binding to DC-SIGN-Fc and compared with the wild type, we observed that binding to DC-SIGN-Fc was significantly affected only in the case of the SlpA-knockout strain, NCK1377-CI. This

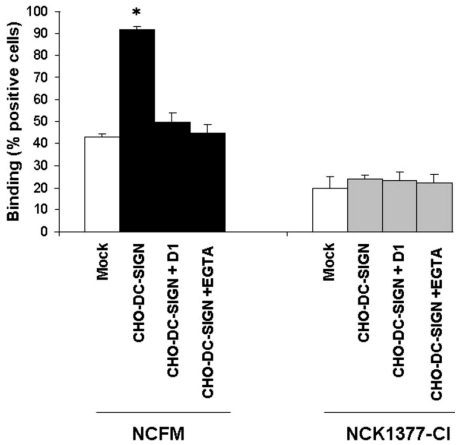


**Fig. 3.** Cellular DC-SIGN is a receptor for *L. acidophilus* NCFM. CHO cells transfected with DC-SIGN and mock (CHO cells alone) ($5 \times 10^4$ cells) were treated with $5 \times 10^6$ FITC-labeled *L. acidophilus* NCFM ($SlpA^+$) or SlpA knockout NCK1377CI ($SlpB^+$) for 45 min at 37 °C followed by FACScan analysis. The DC-SIGN-binding specificity was determined by measuring of the bacterial attachment (percentage positive CHO or CHO–DC-SIGN cells) in the presence of blocking antibody against DC-SIGN, D1 (AZN-D1), and EGTA. The error bars represent standard deviation of data from 3 independent experiments; *, $P < 0.05$.

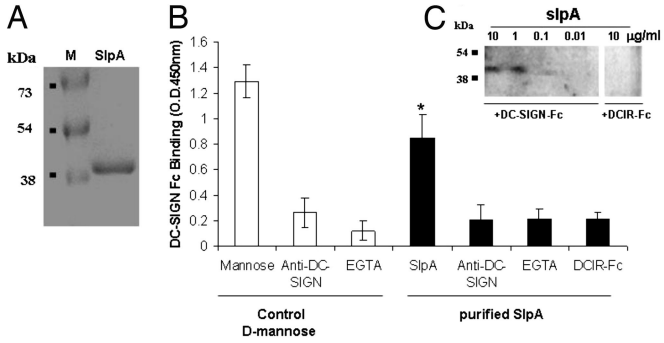


**Fig. 4.** Purified S layer protein ligates to DC-SIGN. After the SlpA purification (*A*), the protein was assayed for its binds to DC-SIGN-Fc by ELISA (*B*). The data are expressed as the mean ± SD of 3 experiments. (*C*) Western blotting results for DC-SIGN-Fc and DCIR-Fc binding to different concentrations of SlpA.

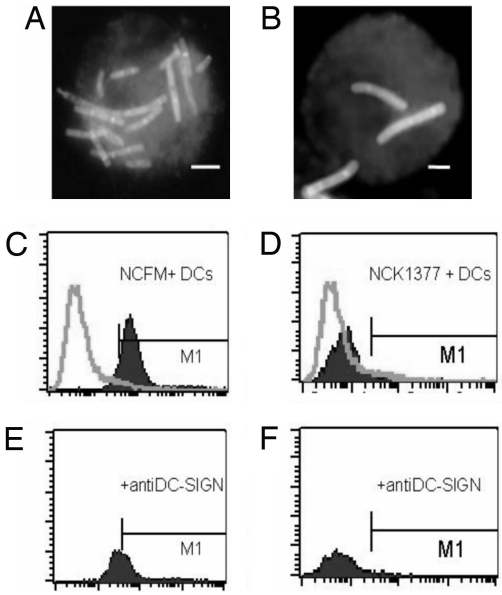


**Fig. 5.** *L. acidophilus* NCFM SlpA mediates binding to DC-SIGN on iDCs. (*A* and *B*) Microscopic analysis of DCs interacting with fluorescently labeled *L. acidophilus* NCFM (*A*) and SlpA-knockout mutant, NCK1377-CI (*B*). (Scale bars, 5 $\mu$m.) (*C* and *D*) FACScan analysis of *L. acidophilus* NCFM or NCK1377-CI binding to iDCs when iDCs ($5 \times 10^4$ cells) were treated with $5 \times 10^6$ FITC-labeled *L. acidophilus* NCFM (SlpA$^+$) (*C*) or SlpA-knockout NCK1377-CI (SlpB$^+$) (*D*) for 45 min at 37 °C. (*E* and *F*) Blocking antibodies to DC-SIGN (AZN-D1, $c$ = 20 $\mu$g mL$^{-1}$) were used when iDCs were treated with *L. acidophilus* NCFM (*E*) or NCK1377-CI (*F*). A representative experiment of 4 is shown.

derivative no longer expresses SlpA because of a chromosomal inversion the predominant surface layer protein expressed is SlpB (ref. 19 and S.L. and T.R.K., unpublished data). Fig. 2 shows DC-SIGN-binding data of NCFM (SlpA$^+$) against NCK1377-CI (SlpB$^+$). Moreover, compared with *L. acidophilus* NCFM, *L. acidophilus* NCK1377-CI binding was significantly lower to CHO cells transfected with DC-SIGN and uninfluenced by the addition of the blocking antibody or EGTA (Fig. 3). These data led us to the hypothesis that SlpA is a ligand for DC-SIGN. Therefore, we purified SlpA from *L. acidophilus* NCFM and confirmed that indeed DC-SIGN-Fc specifically ligated to the purified SlpA and that this could be blocked by the addition of anti-DC-SIGN Ab and EGTA (Fig. 4*B*). The DC-SIGN FC interaction with SlpA was also assessed by Western blots that demonstrated strong binding with the range of 10 to 1 mg of SlpA/mL and a lack of ligation to the control DCIR-Fc (Fig. 4*C*). In contrast, the same concentrations of SlpB, isolated from *L. acidophilus* NCK1377-CI, did not ligate to DC-SIGN-Fc in a Western blotting experiment (data not shown). Because DC-SIGN has specificity for high mannose and fucose, we determined the presence of these carbohydrate moieties on SlpA by using plant lectins, ConA (recognizing high mannose > hybrid-type > biantennary *N*-glycans), and aleuria aurantia lectin (AAL) (specific for $\alpha$6 fucose). ConA strongly ligated to the SlpA, implying the presence of specific glycans after the *Lactobacillus* SlpA purification, whereas AAL showed only a weak binding (Fig. S3).

To evaluate the specific interaction of DC-SIGN with SlpA, we compared the binding of NCFM (SlpA-dominant) and NCK1377-CI (SlpB-dominant) to iDCs. DC-SIGN was involved in the capture of *L. acidophilus* NCFM by the fact that approximately half of the binding *L. acidophilus* NCFM was abrogated by anti-DC-SIGN Ab at ratio 100:1 (probiotic:iDC) (Fig. 5 *C* and *E*). In contrast, attachment of NCK1377-CI (SlpB-dominant) to iDC was significantly lower and unaffected by incubation anti-DC-SIGN Ab (Fig. 5 *D* and *F*). The differential DC ligation of NCFM and NCK1377-CI was also supported by visual examination with fluorescent microscopy, and representative images are shown in Fig. 5 *A* and B, respectively.

In summary, these findings demonstrate that DCs capture *L. acidophilus* NCFM via interactions of DC-SIGN and the surface (S) layer protein, SlpA.

**Interactions of *L. acidophilus* NCFM and Its S layer with TLRs-2 and -4.** Next, we evaluated the potential differences of *L. acidophilus* NCFM and NCK1377-CI to activate TLRs transfected in HEK-293 cells (TLR2 and TLR4 plus MD-2) by determining the level of IL-8 production. PAM3CSK and LPS were analyzed as positive controls for TLR2 and TLR4, respectively. Both NCFM (SlpA dominant) and NCK1377-CI (SlpB dominant) activated the TLR-2 at similar levels and in all ratios examined (1000:1 to 10:1, bacteria:HEK293-TLR2), whereas TLR4 activation was not detected even at the highest doses (Fig. S4). These results indicated that the lack of the S layer protein A expression did not influence the activation of DCs via TLR2 and TLR4.

**Enhanced Production of Proinflammatory Cytokines in iDC Treated with *L. acidophilus* NCK1377 CI Expressing Predominantly SlpB.** A critical function of DCs to produce cytokines was examined in response to stimulation with both NCFM and NCK1377-CI (SlpB-predominant). The production of the antiinflammatory cytokine IL-10, IL-6, and the proinflammatory cytokines IL-12p70, TNF-$\alpha$, and IL-1$\beta$ was assessed at different ratios (bacteria:DC) (Fig. 6). Both strains induced IL-10 at comparable levels in ratios 1,000:1 and 100:1, but the ability of the SlpB-dominant strain to stimulate IL-10 production was reduced significantly at the ratio 10:1 relative to NCFM. To investigate whether SlpA is the structure responsible for the antiinflammatory cytokine profile, we analyzed whether purified SlpA alone or in combination with LPS stimulated higher IL-10 production in iDCs. Indeed, SlpA in combination with LPS induced higher levels of IL-10 compared with LPS or SlpA incubation alone and also led to the production of IL-12p70, TNF-$\alpha$, IL-1$\beta$, and IL-6. Although both bacterial strains (NCFM and NCK1377-CI) were able to stimulate the proinflammatory cytokines such as IL-12p70, TNF-$\alpha$, and IL-1$\beta$ at the highest ratio of bacteria to DC (1,000:1), the SlpB-dominant strain was more potent in the induction of these cytokines at the lower ratios of 100:1 or 10:1. Interestingly, incubation of DCs with *L. acidophilus* NCFM induced a higher IL-6 response compared with the S layer A-knockout at all examined ratios (Fig. 6).

**S Layer of *L. acidophilus* NCFM–DC-SIGN Interaction Is Crucial for the Modulation of T Cell Function.** Next we investigated the potential of purified SlpA (1 $\mu$g/mL), SlpA combined with LPS, *L. acidophi-*

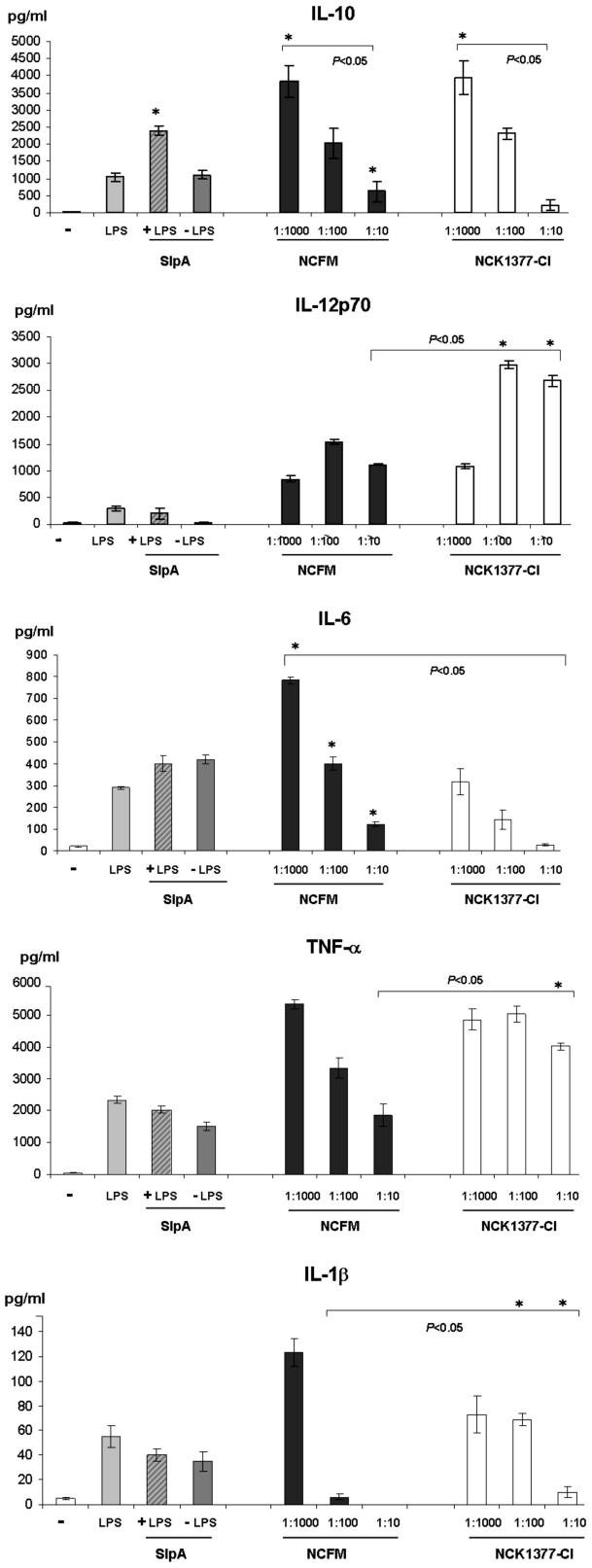


**Fig. 6.** *L. acidophilus* NCFM (SlpA$^+$) and NCK1377-CI (SlpB$^+$) elicit differential iDC cytokine responses. The production of the antiinflammatory cytokines IL-10 and IL-6, and the proinflammatory cytokines IL-12p70, TNF-$\alpha$, and IL-1$\beta$ was analyzed by ELISA at different ratios (DCs:NCFM or DCs:NCK1377-CI) or after the iDCs interactions with a purified SlpA (1 $\mu$g/mL) with and without LPS (10 ng/mL); −, cytokines produced by iDCs left untreated in those experiments; *, significant difference ($P < 0.05$) for the cytokines induced at the respective DC:NCFM or DC:NCK1377-CI ratios.

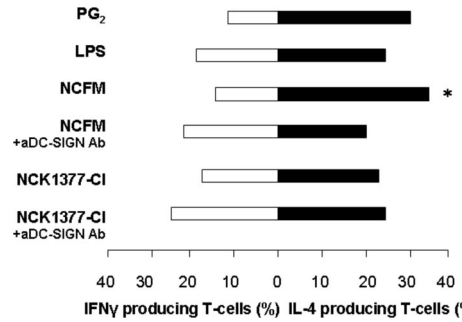


**Fig. 7.** *L. acidophilus* NCFM induces Th2 polarization via C-type DC-SIGN. DCs were maturated with *L. acidophilus* NCFM and the SlpA-knockout, NCK1377CI-(SlpB$^+$) at a ratio of 100:1 (bacteria:DCs), or SlpA+LPS, in the presence or absence of anti-DC-SIGN Ab (aDC-SIGN). The percentage of IL-4- and INF$\gamma$-producing T cells was analyzed upon restimulation of the cultures. The data are the result of 4 independent experiments. *, significant difference ($P < 0.05$) for the cytokines induced after *L. acidophilus* NCFM stimulation.

*lus* NCFM (SlpA-dominant), and NCK1377-CI (SlpB-dominant), at ratio of 100:1 (bacteria:DC), to prime T cells responses after their interactions with DC-SIGN on iDCs. Before the incubation of DCs with T cells, the expression of a CD86 maturation marker on DCs was analyzed (Fig. S5). Purified SlpA led to low levels of CD86 on DCs, whereas a higher abundance of CD86 was detected after the interactions between NCFM, NCK1377-CI, and SlpA in combination with LPS. Further, the matured DCs were washed and incubated with autologous naïve CD4$^+$ T cells, as stated by the expression of CD45RA$^+$ on 90% of the CD4$^+$ T cells, and in ratio $5 \times 10^3$ DCs added to $20 \times 10^3$ naïve T cells, as described in SI *Materials and Methods*. To examine the DC-SIGN involvement in the activation of T cell differentiation, blocking antibodies to DC-SIGN were also used during the maturation of the DCs in the presence of *L. acidophilus* NCFM and NCK1377-CI. DCs stimulated with LPS and $PGE_2$ were cocultured with naïve T cells as a positive control. All cocultures of the mature DCs and naïve T cells were performed for up to 15 days, and the resulting T cells were analyzed for their capacity to secrete IFN$\gamma$ and IL-4, markers for Th1 or Th2 differentiation, respectively. In agreement with previous studies (4, 14), DCs incubated with LPS induce naïve T cell differentiation into a mixed Th1/Th2 response, whereas $PGE_2$ induces a dominant Th2 response (Fig. 7). *L. acidophilus* NCFM was found to induce more IL-4 compared with IFN$\gamma$-producing T cells (34% IL-4 vs. 14% IFN$\gamma$, average of $n = 4$ donors, $P < 0.05$) (Fig. 7). In comparison, a mixed Th1/Th2 response was characteristic for NCK1377-CI (23% IL-4 vs. 18% IFN$\gamma$, $n = 4$ donors). DCs stimulated with the purified SlpA alone did not induce a T cell proliferation. SlpA combined with LPS maturated DCs led to more IL-4-producing T cells isolated from 2 donors and unpolarized response in the case of T cells from another one (data not shown). Blocking of NCFM ligation to DC-SIGN resulted in a mixed response in T cell differentiation. Cytokine ratios in T cells generated in the presence anti-DC-SIGN Ab and NCK1377-CI was not changed. These results demonstrate that DC-SIGN ligation by the SlpA layer of *L. acidophilus* NCFM was crucial for the T cells to acquire their differentiation after bacterial stimulation of DCs.

## Discussion

In the course of the present work, we demonstrated that the cellular contacts of DCs and *L. acidophilus* involve interactions between DC-SIGN and SlpA, the dominant protein expressed by the wild-type strain, NCFM. Insertional inactivation of the *slpA* gene in *L. acidophilus* NCFM led to a chromosomal inversion (19) and expression of a second S layer protein, SlpB, that was

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dominant in NCK1377-CI. In addition, the SlpB-dominant mutant up-regulated production of proinflammatory cytokines. Although both the SlpA-dominant parent strain, NCFM, and the SlpB-dominant mutant activated TLR-2 at similar levels, only NCFM-expressing SlpA was captured to DC-SIGN on DCs, an interaction that appeared to be crucial for the activating of IL-4-producing T cells.

Previous studies have showed that DC-SIGN could play an important role in determining the DC maturation and the outcome of the Th1/Th2 balance. Various bacteria can bind DC-SIGN, including *H. pylori* and mycobacteria (12, 13). Interestingly, ligation of DC-SIGN by mycobacteria and its constituent mannosilated lipoarabinomannan (ManLAM) inhibits LPS-induced DCs maturation and induces IL-10 production (13), whereas *H. pylori* does not inhibit DCs maturation but does induce increased levels of IL-10 in human DCs (12). *N. meningitidis*-expressing *lgtB* LPS also targets DC-SIGN and skewed T cell responses driven by DCs toward Th1 differentiation (15). DC-SIGN could recognize uncharacterized ligands of some commensal bacteria, such as *L. reuteri* and *L. casei*, which, unlike the pathogenic species, neither inhibit DC maturation nor induce increased levels of IL-10 even at high concentrations (4). Although the role of DC-SIGN was not studied extensively in many reports on probiotics, our results have both similarities and differences to previous studies on lactobacilli and human DC interactions (5, 16, 17, 20, 21). In line with the current work, human DCs exposed to lactobacilli, including *L. acidophilus* NCFM, increased CD86 and other costimulatory, adhesion, and activation molecules and induced the expression of IL-10, IL-6, and IL-12 (5, 16). The cytokine profiles of DCs interacting with *L. acidophilus* NCFM and the SlpB-dominant mutant reported here were concentration-dependent. The study clearly demonstrated that IL-10 was up-regulated compared with IL-12p70 at *L. acidophilus* NCFM:DCs ratios of 1,000:1 and 100:1. This implies that a dense *L. acidophilis* NCFM culture is able to stimulate DCs to produce high levels of immune-regulatory IL-10. In contrast, the SlpB-dominant mutant was a potent inducer of proinflammatory cytokines (IL-12p70, TNF$\alpha$, and IL1$\beta$) at lower bacteria:DC ratios (100:1 and/or 10:1). Furthermore, treating DCs with *L. acidophilus* NCFM (SlpA-dominant) abrogated the binding to DC-SIGN by anti-DC-SIGN Ab to 50% at a ratio of 1:100 (Fig. 5). This suggests that other cell surface proteins might also be involved in the NCFM–DC contact. Nevertheless, the functional analyses of the SlpA-knockout mutant and the DC-SIGN-blocking studies have demonstrated that SlpA interaction with DC-SIGN is crucial for the DC cytokine profiles (high IL-10 and IL-6) and the IL-4 expression by T cells. It remains elusive to what extent *L. acidophilus* NCFM-stimulated DCs, which produce high levels of IL-10 and IL-6, are capable of skewing T cells toward Th17 or Treg cells. Moreover, because IL-10 expression is considered a key cytokine for maintaining gut homeostasis and partially responsible for the antiinflammatory effects of probiotic cultures (5), the dose effect of probiotic bacteria in treatment of inflammatory diseases, such as allergy, remains to be established. Our work also suggests that the potential switch between SlpA and SlpB might lead to differential immune responses impacting the gut immune homeostasis. Therefore, further studies need to address at what extent the ratio of SlpA to SlpB present on the surface of different intestinal lactic acid bacteria could influence inflammatory conditions in the GI tract.

The immunoregulatory role of DCs is believed to be determined by ligation of pathogen-recognition receptors such as TLRs and CLRs, and signaling pathways induced by these receptors, which can interconnect through a so-called cross-talk (10, 22). In the present work, the purified SlpA induced an IL-10 production and a low expression of CD86 in DCs, and these DCs were unable to induce a T cell proliferation. This supports the notion that at the given concentration (1 $\mu$g/mL), the SlpA binding to DC-SIGN is not sufficient to induce a strong maturation in DCs. When SlpA was combined with the TLR4 agonist (LPS), however, a higher IL-10 production by DCs, compared with LPS alone, was observed. A similar effect has been described for ManLAM interactions with DC-SIGN (12), indicating that SlpA binding to DC-SIGN could generate an intracellular signal that interferes with the TLR4-mediated DCs activation. Moreover, both the SlpB-dominant mutant and the parent strain stimulated TLR2 to a similar degree, suggesting that the lack of SlpA expression did not influence the *L. acidophilus* NCFM activation of TLR2, which senses peptidoglycan structures conserved among lactobacilli (5, 20). It can be further speculated that the DC-SIGN pathway induced at high bacterial doses overruled the generalized TLR2 immune activation, which prevails at low bacterial loads.

The function of the S layer in *L. acidophilus* NCFM is unknown, but it has been suggested to be important for *Lactobacillus* adhesion to intestinal epithelial cells and extracellular matrix components (18, 23). The S layers of *L. acidophilus* and related species are composed of 1 or more proteins of ≈45 kDa. The carbohydrate moieties interacting with different S layer proteins are currently under investigation and may identify a novel carbohydrate ligand for DC-SIGN.

This work does not provide information on the antigen specificity of the induced T cell population. The in vitro experiments tested *Lactobacillus*-driven DCs maturation and T cell differentiation in polyclonal models by using alloantigen-driven naïve T cell responses and not in an antigen-specific model. This is because of reported difficulties in obtaining sufficient numbers of human antigen-specific, naïve T cells for further analysis (4). It also remains to be established to what extent DCs primed with lactobacilli drive T cell differentiation that are strain-specific and how the bacteria-modulated DCs, as described here, could be generated in different compartments of the intestine.

*L. acidophilus* NCFM has been used for >30 years as a probiotic culture in various dietary supplements and dairy-based foods, predominately yogurt. In vivo, the strain was found to exert antibody- and cell-mediated responses to *Candida albicans* in immunodeficient mice, to play a role in decreasing the severity of candidiasis (2), and to elicit morphine-like effects in the GI tract of mice (24). Probiotic treatment with *L. acidophilus* NCFM also stimulates IL-10 regulatory cytokine expression in the colon and is effective in preventing GI colitis (25). Recently, the DC-SIGN pathway has been demonstrated to be involved in regulation of adaptive immunity by DCs to bacterial, fungal, and viral pathogens (22). The current work establishes that the SlpA-dominant *L. acidophilus* NCFM interacts with a major receptor on DCs and regulates DC immune functions. It suggests that this probiotic bacterium could directly or indirectly interfere with pathogen-induced effects on the host immune system. These data establish a working hypothesis on the mode of action of probiotic cultures that will guide further investigations into the mechanisms by which these bacteria impact GI states.

## Materials and Methods

**Bacterial Strains.** The bacterial strains used in this study are identified in Table S1. All strains were obtained from the stock culture collection maintained in the Department of Food Science at North Carolina State University, Raleigh. Bacterial stock cultures were stored at −20 °C in MRS broth (Difco Laboratories) containing 10% (vol/vol) glycerol. All strains were propagated in MRS broth at 37 °C for 18 h, harvested by centrifugation, washed with PBS (50 mL), and added to iDCs. The number of *L. acidophilus* NCFM was determined by using a published conversation factor ($1.2 \times 10^8$ cfu/mL per $A_{600}$ unit) (26).

**Extraction and Purification of S Layer Protein A and B.** SlpA from *L. acidophilus* NCFM and SlpB from *L. acidophilus* NCK1377-CI were extracted and purified from 100-mL cultures propagated at 37 °C by using a method described in *SI Materials and Methods.* In short, the bacterial pellets were washed with cold

distilled water and suspended in 5 M LiCl at 4 °C followed by stirring for 15 min. The supernatants (containing S layers) were harvested after centrifugation and dialyzed against distilled water. After centrifugation and additional washing step, the S layers were stored at −20 °C before further use.

**ELISA and Western Blotting.** *L. acidophilus* NCFM ($A_{600}$ of 0.1), its mutants (Table S1), and the purified SlpA (5 $\mu$g mL$^{-1}$) or D-mannose (Sigma–Aldrich) were coated on NUNC maxisorb plates (Roskilde) overnight at room temperature. Plates were first blocked with 1% BSA, and then DC-SIGN-Fc or DCIR-Fc (1 $\mu$g mL$^{-1}$) or the biotinylated plant lectins ConA or AAL (Sigma–Aldrich) were added for 2 h at room temperature in the presence or absence of 10 mM EGTA or 20 $\mu$g mL$^{-1}$ AZN-D1 (27). DC-SIGN-Fc binding was detected by using a goat anti-human conjugated with peroxidase (Jackson Immunoresearch), whereas the ligation of the biotinylated lectins was assessed by using peroxidase-labeled avidin (Vector Laboratories). Before Western blotting using DC-SIGN-Fc or DCIR-Fc, varying amounts of purified SlpA or SlpB (10, 1, 0.1, and 0.01 $\mu$g/mL) were electrophoresed on a SDS/polyacrylamide gel followed by blotting to a PVDF membrane (Millipore), as described in ref. 28.

**DC Isolation and CHO-DC-SIGN Culturing.** iDCs were obtained from buffy coats of healthy donors (Sanquin), as described elsewhere (29). In short, human peripheral blood mononuclear cells were isolated by a Ficoll gradient. Monocytes were isolated by CD14 magnetic microbeads (MACS; Miltenyibiotec) and differentiated into iDCs in the presence of IL-4 and GM-CSF (500 and 800 units/mL, respectively; Biosource). iDCs expressed high levels of major histocompatibility complex classes I and II, CD11b, CD11c, and ICAM-1 and low levels of CD80 and CD86, confirmed by flow cytometry and defined as iDCs. CHO and CHO transfected with DC-SIGN were maintained in RPMI or DMEM (Invitrogen) containing 8–10% FCS.

**DC Maturation, Cytokine Measurements, and DC-Driven $T_H1/T_H2$ Differentiation.** Immature monocyte-derived DCs (day 6) were stimulated with different concentrations of *L. acidophilus* NCFM, the SlpA-knockout strain, NCK1377-CI (SlpB-dominant), or the purified S layer preparations from NCFM (SlpA-dominant, 1 $\mu$g/mL) in the presence or absence of LPS (10 ng/mL; *Salmonella typhosa*, Sigma–Aldrich) for 24 h at 37 °C. Cell surface expression of costimulatory molecules CD86 (BD PharMingen) using phycoerythrin-conjugated antibodies was used to determine maturation. Cytokines were measured in the DCs culture supernatants by ELISA with CytoSets ELISA kits (Biosource) for human IL-6, IL-10, TNF-$\alpha$, and IL-1$\beta$ according to the manufacturer's instructions. Human IL-12p70 detection was determined as described in ref. 28.

Before the Th1/Th2 differentiation, iDCs were matured for 2 days with *L. acidophilus* NCFM, NCK1377-CI, and slpA (1 $\mu$g/mL) with and without LPS (10 ng/mL). Subsequently, DCs were incubated with autologous CD45RA$^+$/CD4$^+$ T cells (naïve T cells) at a ratio of 1:4 (DCs:T cells). After 15 days and the application of a specific stimulation protocol, the production of IL-4 and INF$\gamma$ in T cells was determined by FACs. The details of the DC-driven Th1/Th2 differentiation procedure are fully described in ref. 29 and in *SI Materials and Methods*.

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