

PsL-EGFmAb inhibits the stimulatory functions of human dendritic cells via DC-SIGN

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1. ABSTRACT

Dendritic cell (DC)-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) is a DC-specific C-type lectin that plays an important role in recognizing and capturing pathogens, DC migration and initiation of T cell responses. Here, we show that anti-P-selectin lectin-EGF domain monoclonal antibody (PsL-EGFmAb), originally prepared for blockade of the adhesive molecule P-selectin, significantly down-regulated DC-SIGN expression as well as expression of mature DC-related molecules including CD83, CD86 and CD80 on human DCs. This PsL-EGFmAb treatment of DCs resulted in impaired allogeneic T cell proliferation and IL-12 production. Furthermore, we show that PsL-EGFmAb-induced down-regulation of DC-SIGN may inhibit NF-kappaB expression in DCs, which accounts for the inhibition of DC maturation and stimulatory function. Our present studies indicate that PsL-EGFmAb may be a useful reagent for regulating DC-SIGN expression and DC function.

2. INTRODUCTION

Dendritic cells (DCs) are specialized antigen presenting cells with a unique ability to activate naive T cells. Therefore, they play a pivotal role in the regulation of innate and adaptive immune responses (1,2). A fundamental aspect of DC function that controls immunity is the ability of DCs to migrate (3). Immature DCs can migrate from circulating blood to inflammatory tissues in association with P-selectin, E-selectin and DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), where they patrol for foreign antigens (4,5,6).

P-selectin is a member of the selectin family and is predominantly expressed on activated endothelial cells and activated blood platelets. The selectin family also includes E-selectin and L-selectin. These selectins are transmembrane glycoproteins and belong to the C-type lectin family (7). P-selectin contains two major domains, lectin and epidermal growth factor (EGF). The lectin domain is involved in ligand recognition and

mediation of cell-cell adhesion (8). The EGF domain regulates and stabilizes the conformation of P-selectin, thereby enhancing ligand binding affinity and specificity. Together, the lectin and EGF domains of P-selectin constitute the optimal functional domain necessary for leukocyte adhesion (8,9). Accordingly, we prepared an anti-P-selectin lectin-EGF domain monoclonal antibody (PsL-EGFmAb) that displayed a better adhesion blocking effect than anti-P-selectin mAb (10,11). We have previously shown that PsL-EGFmAb inhibited not only DC migration to inflammatory tissue *in vivo*, but also the maturation of immature DCs *in vitro* (12). These studies suggest that PsL-EGFmAb regulates DC maturation and function in addition to blocking cell adhesion and DC migration.

Accumulating evidences have shown that the immunoregulatory function of DCs is mediated by cell-surface molecules such as Toll-like receptors and C-type lectins (13). The C-type lectin, DC-SIGN, first identified as a co-stimulatory molecule in DC-T cell interactions (14), is a pattern recognition receptor (PRR) and adhesion receptor of DCs. As a PRR, DC-SIGN can mediate the capture and internalization of viral, bacterial and fungal pathogens by myeloid DCs (13). Moreover, it can also be exploited by some pathogens such as HIV-1 and *Mycobacterium tuberculosis* to escape immune surveillance (15,16). As an adhesion receptor, DC-SIGN interacts with intercellular adhesion molecule (ICAM)-2 on endothelial cells to mediate DC migration. It also interacts with ICAM-3 on T cells to initiate a T cell immune response (6,14). These studies emphasize the important role of DC-SIGN in DC functions, and further suggest that DC-SIGN is potentially a critical target molecule in the treatment of inflammatory and infectious diseases.

As a transmembrane glycoprotein, DC-SIGN has an extracellular domain that contains a C-type lectin C-terminal carbohydrate-recognition domain (CRD), similar to the C-type lectin CRD of selectins (17,18). As both P-selectin and DC-SIGN possess a lectin domain, and since PsL-EGFmAb specifically recognizes the lectin domain, we examined whether PsL-EGFmAb could regulate DC-SIGN expression and DC functions. Interleukin (IL)-10 is a potent regulator of DC maturation and functions, and can suppress the expression of co-stimulatory molecules on DCs (19). Thus, IL-10 was considered to be a suitable control reagent in the present study. We found that PsL-EGFmAb down-regulated DC-SIGN expression on DCs, leading to impairment of DC maturation and stimulatory functions. In addition, nuclear transcription factor-kappaB (NF-kappaB) was decreased in PsL-EGFmAb-treated DCs. These results suggested that PsL-EGFmAb-induced down-regulation of DC-SIGN may contribute to the decreased expression of NF-kappaB in DCs, and consequently lead to the inhibition of DC maturation and stimulatory functions. These findings suggest that PsL-EGFmAb may be a useful tool for inhibiting DC-SIGN expression and DC function.

3. MATERIALS AND METHODS

3.1. Reagents and antibodies

Human stem cell factor (SCF), granulocyte-macrophage colony stimulating factor (GM-CSF), transforming growth factor-beta1 (TGF-beta1), Fms-like tyrosine-kinase-3 ligand (Flt3L) and tumor necrosis factor-alpha (TNF-alpha) were purchased from Biosource (Camarillo, CA). Anti-human magnetic bead-labeled CD34 and CD4 antibodies were purchased from Miltenyi Biotec (Aulburm, CA). Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD83 and CD86, phycoerythrin (PE)-conjugated mouse anti-human CD80, Cy-chrome (CY)-conjugated mouse anti-human CD1a and HLA-DR mAbs were obtained from BD Bioscience (San Diego, CA). PE-conjugated mouse anti-human CD11c and CD54 mAbs were obtained from Biosource. PE-conjugated mouse anti-human P-selectin, CY-conjugated mouse anti-human L-selectin, FITC-conjugated mouse anti-human DC-SIGN mAbs and FITC-labeled rat IgG were obtained from eBiosource (San Diego, CA). Goat anti-mouse Ig-R-PE, Ig-FITC and Ig-CY were from Southern Biotech (Birmingham, AL). FITC-conjugated mouse anti-human E-selectin mAb was obtained from Ancell (Bayport, MN). 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide (MTT) was from Sigma (St. Louis, MO). Trizol used for RNA extraction was purchased from Invitrogen (Carlsbad, CA). TaqE, dNTP and reverse transcription polymerase chain reaction (RT-PCR) kits were purchased from Promega (Madison, WI). The IL-12p70 enzyme linked immunosorbent assay (ELISA) kit was purchased from Biosource. PsL-EGFmAb was prepared in our laboratory (10).

3.2. Cells

Human cord blood was obtained from healthy volunteers from the Department of Obstetrics, Ruijin Hospital (Shanghai, China). Mononuclear cells (MNCs) were separated from the cord blood by Ficoll-Hypaque gradient centrifugation. CD34⁺ cells were first isolated from MNCs through magnetic bead-labeled anti-human CD34 mAb, then cultured at 0.5×10^6 cells/ml in 24-well plates in Iscove's modified Dulbecco's medium (Gibco, Rockville, MD) containing 20% fetal bovine serum, 50 ng/ml SCF, 100 ng/ml GM-CSF, 0.5 ng/ml TGF-beta1 and 75 ng/ml Flt3L for 7 days to induce immature DCs. Immature DCs were stimulated with PsL-EGFmAb (5, 10, 50, 100 µg/ml) or IL-10 (10, 50, 100, 200 ng/ml) for an additional 7 days. TNF-alpha (100 ng/ml) was added to all the cultures at day 12 to induce DC maturation. Immature DCs were harvested at day 7, PsL-EGFmAb- or IL-10-treated DCs and mature DCs were collected at day 14 for various testing.

3.3. DC phenotypic analysis

Phenotypic analysis was performed by flow cytometry using a FACS Calibur and CellQuest software (Becton Dickinson, San Jose, CA). Briefly, 5×10^5 cultured cells were stained with PE-, CY- and FITC-labeled mAbs specific for HLA-DR, CD1a, CD11c, CD54, CD83, CD86 and DC-SIGN. Goat anti-mouse Ig-R-PE, Ig-FITC and Ig-CY were used as isotype controls.

3.4. Mixed lymphocyte reaction (MLR)

Allogeneic CD4⁺ T cells obtained from peripheral blood MNCs were used as responders and prepared with magnetic bead-labeled anti-human CD4 mAb. DCs were exposed to X-ray irradiation (30 Gy) and seeded in 96-well plates as stimulators (3×10⁴ cells/well). CD4⁺ T cells were added to each well, followed by incubation at 37 °C for 5 days. Cellular proliferation was determined using the MTT assay. Briefly, 15 µl of MTT (5 mg/ml) was added to each well, and the plates were incubated at 37 °C for 4 h. The resultant absorbance was read at 550 nm by a microplate immunoreader.

3.5. Analysis of IL-12p35 and IL-12p40 expression

Total RNA was isolated from immature DCs, mature DCs and PsL-EGFmAb- or IL-10-treated DCs using the RNA Trizol kit according to the manufacturer's instructions. cDNA was synthesized from 3.0 µg of total RNA using oligo (dT) primers and a RT-PCR kit. The IL-12p35 primer sequences were as follows: sense 5'-TCTCCATTCCCCATTCTCAG-3'; antisense 5'-ATGTGGCAGAATTTGCATGA-3'. The amplified products were 200 bp. The IL-12p40 primer sequences were as follows: sense 5'-CATGGGCCTTCATGCTATTT-3'; antisense 5'-TGATGTACTTGCAGCCTTGC-3'. The amplified products were 203 bp. The primers for beta-actin were as follows: sense 5'-TCGTATCCCTGTACGCCTCT-3'; antisense 5'-AGTACTTGCGCTCAGGAGGA-3'. The amplified products were 597 bp. The PCR reaction was as follows: an initial melting procedure at 95 °C for 5 min; then 34 cycles of amplification of 1 min at 94 °C, 40 s at 60 °C, and 1 min at 72 °C; finally extension at 72 °C for 10 min. The PCR products were visualized by ethidium bromide staining following resolution on a 2% agarose gel.

3.6. ELISA

The supernatants were collected from cultures of immature DCs, mature DCs and PsL-EGFmAb- or IL-10-treated DCs. The production of IL-12p70 was determined by an IL-12p70 ELISA kit according to the manufacturer's instructions.

3.7. Assessment of NF-kappaBp50 and NF-kappaBp56 mRNA levels

Total RNA was isolated and cDNA was synthesized as described above. The NF-kappaBp50 primer sequences were as follows: sense 5'-GACCAAGGAGATGGACCTCA-3'; antisense 5'-CGAAGCTGGACAAACACAGA-3'. The amplified products were 200 bp. The NF-kappaBp56 primer sequences were as follows: sense 5'-TCAATGGCTACACAGGACCA-3'; antisense 5'-ATCTTGAGCTCGGCAGTGTT-3'. The PCR reaction was conducted as described above.

3.8. Statistical analysis

The data are expressed as the mean±SD. *P* values were determined by one-way ANOVA and the Student-Newman-Keuls test was used for multiple group comparisons. *P* < 0.05 was considered to be statistically significant.

4. RESULTS

4.1. PsL-EGFmAb down-regulates the expression of C-type lectin DC-SIGN on DCs

To examine whether PsL-EGFmAb regulates the expression of DC-SIGN, we first determined the level of selectin expression on DCs. P-selectin and E-selectin expression was undetectable on immature and mature DCs. Moderate levels of L-selectin expression were observed on immature DCs, but this was reduced to undetectable levels as the DCs matured (Figure 1A).

Next we examined the expression of DC-SIGN on DCs. Immature DCs collected at day 7 were stimulated with PsL-EGFmAb for an additional 7 days. Cells were collected at day 12 and cultured in the presence of TNF-alpha for an additional 2 days to induce DC maturation. Treatment with PsL-EGFmAb did not affect the proliferation, expansion, viability, and generation of immature DCs (data not shown). Immature and mature DCs displayed similarly high levels of DC-SIGN. The level of DC-SIGN expression was markedly reduced on PsL-EGFmAb-treated DCs compared with control IgG-treated-DCs (Figure 1B and data not shown). However, this effect was not mediated by IL-10 since DC-SIGN expression was unaffected by IL-10 treatment (Figure 1B). These findings suggest that PsL-EGFmAb inhibits the expression of DC-SIGN on DCs.

4.2. PsL-EGFmAb inhibits DC maturation

We next examined whether DC maturation was affected by the down-regulation of DC-SIGN after PsL-EGFmAb treatment. Mature DCs showed increased expression of the co-stimulatory molecules, CD80 and CD86, and the mature DC marker CD83, as well as HLA-DR compared with immature DCs. PsL-EGFmAb-treated DCs, however, showed a significant reduction in CD80, CD86 and CD83 expression, while the expression of HLA-DR remained virtually unchanged. It has previously been demonstrated that IL-10 inhibits DC maturation (20). We found that addition of IL-10 also inhibited DC maturation, and that this effect was similar to that observed after PsL-EGFmAb treatment (Figure 2). These results suggest that PsL-EGFmAb inhibits the phenotypic maturation of DCs.

4.3. PsL-EGFmAb-treated DCs are functionally defective in activating naive T cells and IL-12 production

To determine whether PsL-EGFmAb treatment affects the stimulatory functions of DCs, we examined the ability of DCs to stimulate allogeneic T cell proliferation after treatment with PsL-EGFmAb. As expected, mature DCs stimulated T cell proliferation, whereas immature DCs failed to exert this stimulatory effect. After treatment with PsL-EGFmAb or IL-10, DCs were much less potent to stimulate allogeneic T cell proliferation compared with control mature DCs (Figure 3A). These findings suggest that PsL-EGFmAb suppresses DC-mediated stimulation of T cell proliferation.

IL-12 is a potent DC-derived factor required for Th1 differentiation (21). To examine further the effects of

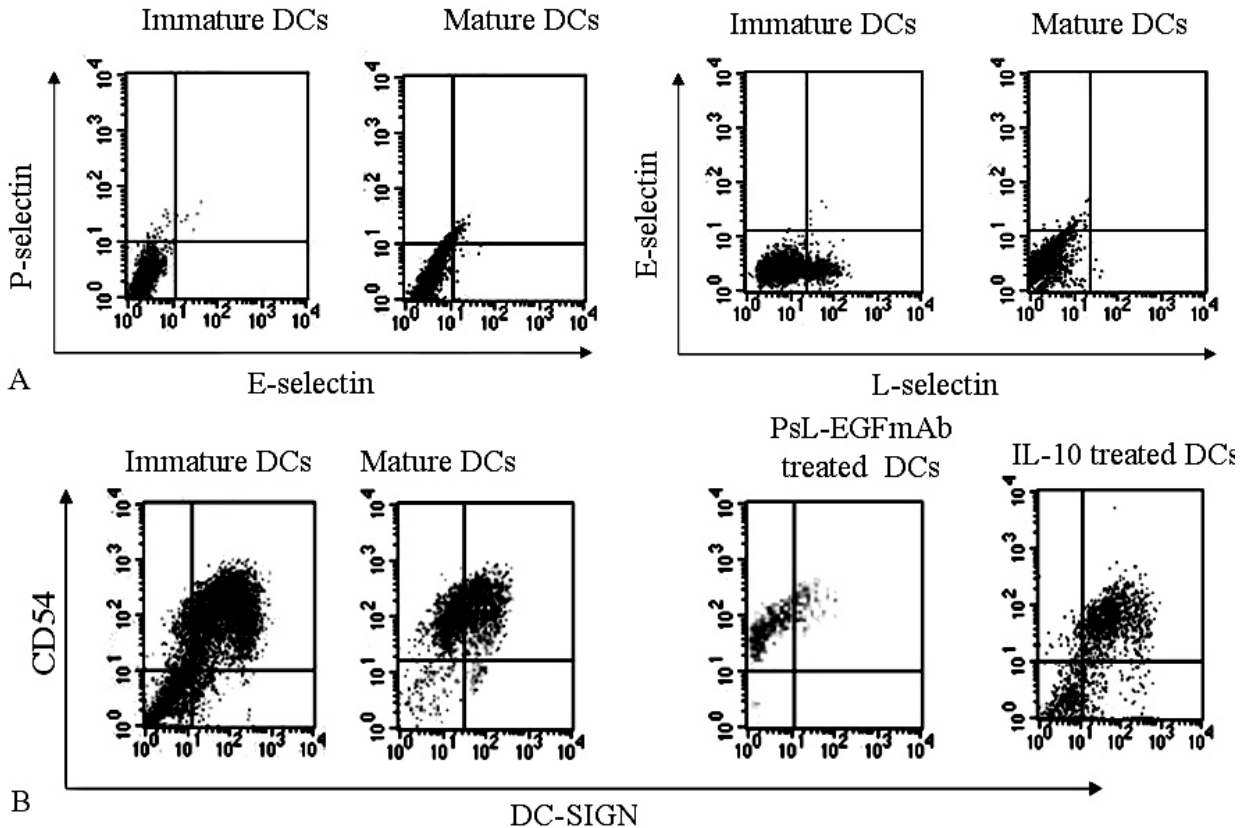


Figure 1. Expression of selectins and DC-SIGN on DCs. Immature DCs were collected at day 7 and incubated with PsL-EGFmAb and IL-10 for 7 days, respectively. Two days before harvesting, TNF-alpha was added to induce DC maturation. Expression of selectins and DC-SIGN were analyzed by FACS. (A) Expression of P-, E- and L-selectin on immature and mature DCs. (B) Expression of DC-SIGN on immature DCs, mature DCs, PsL-EGFmAb- and IL-10-treated DCs. Results are representative of three independent experiments.

PsL-EGFmAb on DC functions, we measured the mRNA levels of IL-12p35 and IL-12p40 by RT-PCR. Mature DCs expressed higher levels of IL-12p35 and IL-12p40 mRNA than immature DCs (Figure 3B). PsL-EGFmAb-treated DCs produced significantly less IL-12p35 and IL-12p40 compared with control mature DCs. This inhibitory effect of PsL-EGFmAb on IL-12 expression was dose-dependent and similar to that observed after treatment with IL-10 (Figure 3B). These results were further confirmed by ELISA. We found that mature DCs produced significantly higher IL-12p70 levels in the supernatant than immature DCs (358.24 ± 23.55 pg/ml vs. 0.37 ± 0.03 pg/ml), whereas PsL-EGFmAb-treated DCs produced significantly lower IL-12p70 levels (12.34 ± 8.56 pg/ml) than mature DCs. IL-10-treated DCs induced a marginal inhibitory effect on the IL-12p70 production of DCs (165.42 ± 137.68 pg/ml). These data suggest that the inability of PsL-EGFmAb-treated DCs to stimulate T cell proliferation and differentiation may be associated with its ability to suppress IL-12 production.

4.4. PsL-EGFmAb inhibits the expression of NF-kappaBp50 and NF-kappaBp65 in DCs

The NF-kappaB signal transduction pathway plays an important role in regulating DC functions through

DC-SIGN (22). To further explore the possible mechanism of PsL-EGFmAb-mediated inhibition of DC maturation and stimulatory functions, we examined the mRNA expression of NF-kappaBp50 and NF-kappaBp65 in DCs. RT-PCR analysis revealed increased levels of NF-kappaBp50 and NF-kappaBp65 mRNA in mature DCs compared with immature DCs. However, pretreatment with PsL-EGFmAb or IL-10 suppressed the mRNA levels of NF-kappaBp50 and NF-kappaBp65 in mature DCs (Figure 4). These data suggest that PsL-EGFmAb-induced down-regulation of DC-SIGN may inhibit the expression of NF-kappaB in DCs. It is likely that inhibiting NF-kappaB via DC-SIGN leads to the reduced DC maturation and stimulatory functions.

5. DISCUSSION

DC-SIGN has many important roles in mediating DC adhesion, migration, inflammation, activating primary T cells, triggering immune responses and participating in the immune escape of pathogens and tumors (23). DC-SIGN is a C-type lectin receptor that can recognize high-mannose glycans and fucose-containing structures present on the surface of pathogens and self-antigens (24). Binding of DC-SIGN to pathogens leads to antigen uptake and

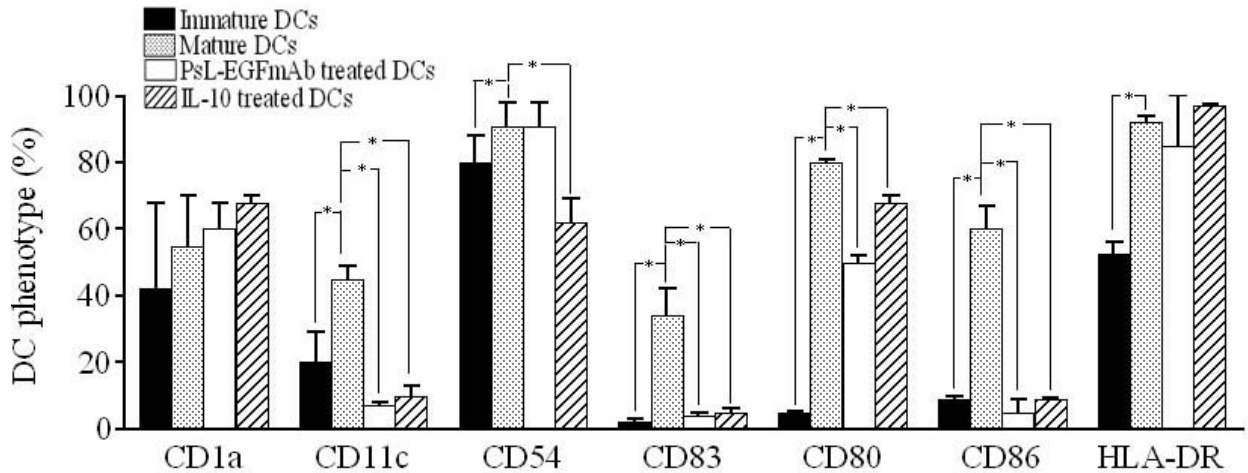


Figure 2. Immunophenotyping characteristics of DCs. DCs were generated and analyzed by flow cytometry as described in Material and methods. PsL-EGFmAb or IL-10 was added to the cultured DCs at day 7, and additional TNF-alpha was added at day 12 to induce DC maturation. Cells were harvested for analysis at day 14. Bars represent mean±SD in triplicate (* $P < 0.05$).

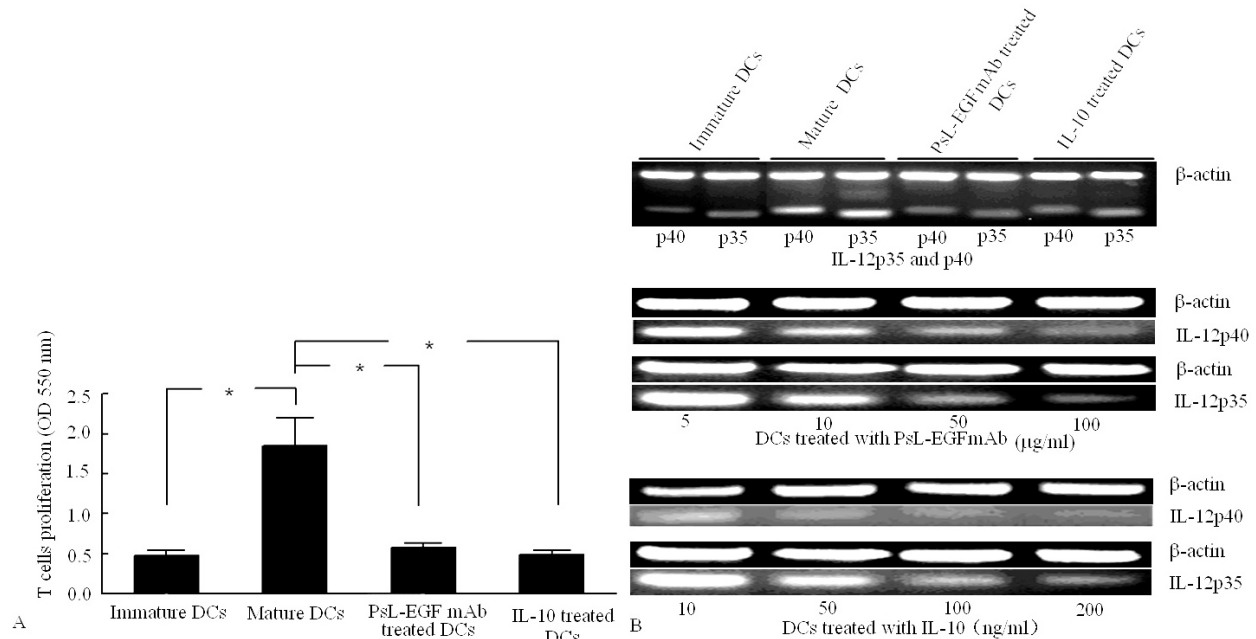


Figure 3. The functions of DCs were suppressed by PsL-EGFmAb. (A) PsL-EGFmAb-treated DCs were unable to induce allogeneic T cell proliferation in MLR. Allogeneic MLR was performed using CD4⁺ T cells from human MNCs as responder cells. Irradiated DCs were used as stimulator cells (3×10^4 cells/well). Proliferation of T cells was measured using the MTT assay after 5 days of culture. Results are expressed as the mean±SD of triplicate cultures from three experiments (* $P < 0.05$). (B) IL-12p35 and IL-12p40 production of DCs were inhibited by PsL-EGFmAb. RNA was extracted from cultured cells using a RNA Trizol kit and RT-PCR was performed as described in Materials and methods. Results are representative of three independent experiments.

processing by DCs (25). DC-SIGN is also an important adhesive receptor that can interact with ICAM-2 and ICAM-3 to mediate DC trafficking and initiation of T cell immune responses (6,14). Previous studies have established that the critical domain for DC-SIGN function is the extracellular C-type lectin CRD, which can recognize certain carbohydrate-contained antigens and mediate the

binding between DCs and pathogens (26,27). PsL-EGFmAb is a monoclonal antibody that specifically recognizes the lectin domain of P-selectin (10). In this study, we show that DCs treated with PsL-EGFmAb exhibit a lower level of DC-SIGN expression than untreated DCs. However, DC-SIGN expression was unaffected by IL-10 treatment. Since treatment with PsL-

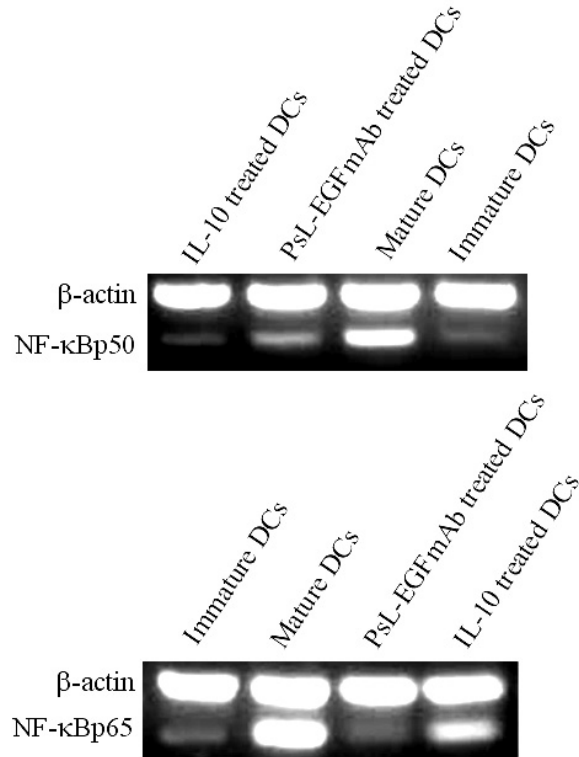


Figure 4. Expression of NF-kappaBp50 and NF-kappaBp65 mRNA in DCs. RNA was extracted from cultured cells and RT-PCR was performed as described in Materials and methods. The data shown are representative of three independent experiments.

EGFmAb did not affect the proliferation, expansion and viability of DCs, it is unlikely that the decreased DC-SIGN expression was due to any toxic side-effects of the PsL-EGFmAb. Since DC-SIGN possesses the same lectin domain as P-selectin, it will be intriguing to further determine whether PsL-EGFmAb can block DC-SIGN.

Previous studies have shown that ligation of DC-SIGN with a stimulatory antibody can regulate DC maturation and functions (28). Since DC-SIGN possesses a lectin domain and unlike selectins is expressed abundantly on mature DCs, we hypothesize that the PsL-EGFmAb may target DC-SIGN to modulate DC functions in the present study. Our data suggest that PsL-EGFmAb down-regulated DC-SIGN and subsequently blocked DC maturation, as evidenced by the decrease in the levels of co-stimulatory molecules. We also found that PsL-EGFmAb-treated DCs were unable to trigger T cell proliferation as potent as control mature DCs. Data from previous studies have shown that DC-SIGN mediates DC-T cell clustering and DC-induced proliferation of resting T cells (13). We propose that a reduction in DC-SIGN expression together with inhibition of DC maturation may contribute to impaired DC-induced T cell proliferation.

A balance between Th1/Th2 responses is important in determining the outcome of inflammation and

related diseases. IL-12 regulates differentiation of naive T cells into Th1 cells, which play a vital role in induction of inflammation. We assessed IL-12 production in PsL-EGFmAb-treated DCs, and found that PsL-EGFmAb inhibited the level of IL-12 expression in a dose-dependent manner. This result further indicates that PsL-EGFmAb has a negative effect on DC-induced Th1 immune responses.

DC-SIGN modulates DC maturation as well as cytokine production and plays an important role in DC-induced immune responses (16,23), probably by regulating NF-kappaB activity (22). We found that the expression of NF-kappaB was decreased in PsL-EGFmAb-treated DCs. A recent study found that DC-SIGN could induce phosphorylation of extracellular signal-regulated kinase (ERK) and protein kinase B (PKB) (28). Moreover, PKB activation could up-regulate NF-kappaB expression (29). Thus, we suggest that PsL-EGFmAb induced down-regulation of DC-SIGN resulting in decreased activation of PKB and reduced expression of NF-kappaB, leading to inhibition of DC maturation and stimulatory functions. Furthermore, PsL-EGFmAb-treated DCs exhibited high levels of HLA-DR expression, low levels of co-stimulatory molecule expression, and failed to stimulate allogeneic T cell proliferation, implying that PsL-EGFmAb-treated DCs may be tolerogenic. However, the underlying mechanisms of tolerogenic DC induction by PsL-EGFmAb are still unknown. Thus, further studies are required to elucidate them.

In summary, we found that PsL-EGFmAb down-regulated the expression of DC-SIGN on DCs derived from human cord blood, leading to inhibition of DC maturation and stimulatory functions. Decreased expression of NF-kappaB resulting from decreased DC-SIGN expression may contribute to the inhibitory effects of PsL-EGFmAb. Our study suggests that PsL-EGFmAb may be a useful tool for regulating DC-SIGN and DC function.

6. ACKNOWLEDGEMENTS

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Abbreviations: DC: dendritic cell; DC-SIGN: dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin; PsL-EGFmAb: anti-P-selectin lectin-EGF domain monoclonal antibody; EGF: epidermal growth factor; PRR: pattern recognition receptor; ICAM: intercellular adhesion molecule; CRD: carbohydrate-recognition domain; Interleukin (IL), NF-kappaB: nuclear transcription factor-kappaB; SCF: stem cell factor; GM-CSF: granulocyte-macrophage colony stimulating factor; TGF-beta1: transforming growth factor-beta1; Flt3L: Fms-

PsL-EGFmAb inhibits DC stimulatory functions

like tyrosine-kinase-3 ligand; TNF- α ; tumor necrosis factor- α ; FITC: fluorescein isothiocyanate; PE: phycoerythrin; CY: Cy-chrome; MTT: 3-(4,5-dimethyl thiazolyl-2-)-2,5-diphenyl-tetrazolium bromide; RT-PCR: reverse transcription-polymerase chain reaction; ELISA: enzyme linked immunosorbent assay; MNCs: mononuclear cells; MLR: mixed lymphocyte reaction; ERK: extracellular signal-regulated kinase; PKB: protein kinase B

Key Words: PsL-EGFmAb, DC-SIGN, C-type lectin, dendritic cells

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