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Ral-Binding Protein is Required for the Maturation and Function of Dendritic Cells

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Abstract: Problem statement: Dendritic Cells (DCs) integrate responses of innate and adaptive immune system via transmitting pro-inflammatory and regulatory signals to naïve cells within draining lymph nodes. DCs have high capacity for endocytosis, both Clathrin-Dependent (CDE) and Caveolin-Dependent (CvDE). **Approach:** We have recently shown that ATP-hydrolysis by the multifunctional transporter protein RLIP76 is the primary determinant of the rate of CDE. Activation of CDE is necessary for DCs to internalize and process exogenous antigens. Thus, in present studies we investigated the role of RLIP76 in antigen uptake, maturation and T cell priming capacity of monocyte-derived DCs. **Results:** Using flow cytometry, we demonstrate variable surface expression of RLIP76 on immature DCs generated from peripheral blood mononuclear cells from healthy individuals. Studies on the effect of anti-RLIP76 antibodies on endocytosis of DC-specific C-type Lectin Receptors (CLRs) (DC-SIGN and DEC-205) and on co-localization of these receptors with MHC-class II were conducted using confocal microscopy. RLIP76-specific antibodies suppressed the maturation of DCs and the expression of typical activation markers and co-stimulatory and adhesion molecules including CD83, HLA-DR, HLA-ABC, CD40, CD80 and CD38. They also inhibited the stimulating potency of DCs in allogeneic Mixed Leukocyte Reaction (allo-MLR). **Conclusions:** We conclude that RLIP76 is necessary for activation, membrane trafficking and functional maturation of DCs. Further exploration of the role of RLIP76 in DC biology is warranted and may provide promising means in DC-based immunotherapies.

Key words: Dendritic cells, RLIP76, DC-SIGN, Caveolin-Dependent Endocytosis (CvDE), DC-based immunotherapies, C-type Lectin Receptors (CLRs), Pattern-Recognition Receptors (PRRs), Antigen Presenting Cells (APCs), oxidative stress, embryonic fibroblasts, stimulating potency

INTRODUCTION

Antigen presentation is an important function of the immune system for initiating protective immune response against foreign and/or harmful endogenous structures and in maintaining self-tolerance. Dendritic Cells (DCs), together with monocytes and macrophages constitute the heterogeneous population of mononuclear phagocytic system and act as non-specific effectors against foreign intruders and tumors (Banchereau and Steinman, 1998; Mellman and Steinman, 2001; Steinman and Banchereau, 2007). In their immature state, DCs localized throughout peripheral tissues recognize Pathogen-Associated Molecular Patterns (PAMPs) and, through an array of Pattern-Recognition Receptors (PRRs) capable of binding and internalizing ligands of diverse structures (Janeway, Jr. and Medzhitov, 2002; Yu *et al*., 2007), channel activation/maturation signals into the cells for their further differentiation. Immature DCs (iDCs), in addition to macropinocytosis and phagocytosis, capture antigens by receptor-mediated endocytosis (Shi *et al*., 2008; Sakr, 2010), including Clathrin-Dependent (CDE) and Caveolin-Dependent Endocytosis (CvDE). DCs receive simultaneous activation signals through the binding of conserved molecular motifs leading to their

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maturation and migration to secondary lymphoid organs (Sallusto and Lanzavecchia, 1999; Flores-Romo, 2001; Block and Markovic, 2009). There, DCs present processed antigens to naïve T cells, thereby inducing antigen specific adaptive immune response (Schumann, J., 2010; Block and Markovic, 2009). This unique capacity of DCs renders them the most potent professional Antigen Presenting Cells (APCs). CDE is a crucial cellular mechanism that regulates membrane trafficking. RLIP76 is a multifunctional modular protein, the first known effector of Ral and has been proposed as a link between Ral and Ras signaling. In addition to its GAP activity towards Cdc42/Rac1 and Rho, RLIP76 is the chief transporter of Glutathione-Electrophile (GS-E) conjugates (Cantor *et al*., 1995; Jullien-Flores *et al*., 1995; Park and Weinberg, 1995; Awasthi *et al*., 2000), precursors of mercapturic acid. Cdc42, in mast cells, plays an important role in biosynthesis of lipid raft components (Field *et al*., 2000), which are critically involved in signal transduction during immune response initiation (Simons and Toomre, 2001; Krishnaraju *et al*., 2009). Available data on small GTPase expression and function in DCs suggest that Cdc42 and Rac1 activity are critical for polarization and motility of monocyte-derived DCs (Burns *et al*., 2001; Swetman *et al*., 2002; Block and Markovic, 2009). RLIP76 is known to interact with μ2, the medium chain of AP2 complex that recruits motor proteins to clathrin-coated pits (Jullien-Flores *et al*., 2000; Awasthi *et al*., 2000; Singhal *et al*., 2008a; Oshiro *et al*., 2002). Receptors endocytosed in clathrin-coated pits display a cytoplasmic "endocytic" tyrosine-based YXXΦ or YXXL motif (Engering *et al*., 2002) and these signals are recognized by μ2. The μ2/RLIP76 interaction is thought to modulate the ability of μ2 to bind YXXΦ-containing proteins and thus membrane association of AP2 and the function of clathrin-coated pits. We recently discovered that RLIP76 knockout mice are entirely deficient in CDE (Singhal *et al*., 2008b); though this CDE can be fully reconstituted by transfecting wild-type RLIP76 into mouse embryonic fibroblasts, mutants of RLIP76 lacking GS-E binding- or nucleotidase activity fail to reconstitute CDE. RLIP76 also provides defense against oxidative stress by transporting glutathione conjugates of electrophilic lipid peroxidation toxic end-products, e.g., 4-hydroxynonenal, generated during oxidative stress (Awasthi *et al*., 2008; Mucimapura *et al*., 2010; Yu *et al*., 2007). In mammalian cells, RLIP76 accounts for up to 80% transport of glutathione conjugates of drugs and endogenous electrophiles (Awasthi *et al*., 2003; 2002; Singhal *et al*., 2003) and in human cells it has been shown to be the major determinant of drug resistance (Awasthi *et al*., 2006). There is mounting evidence that a crucial link exists between the transport function of RLIP76, receptor-ligand endocytosis and various signaling pathways (Awasthi *et al*., 2003; Yadav *et al*., 2007; Shi *et al*., 2008). This would suggest a possible role of RLIP76 in antigen uptake and processing by APCs.

Based on the multiple functions of RLIP76 highlighted above (Singhal *et al*., 2008a; Yadav *et al*., 2007; Nakashima *et al*., 1999) and its functional and substrate profile similarities with other drug transporters (Skazik *et al*., 2008; Robbiani *et al*., 2000; Van De Ven *et al*., 2006; Laupèze *et al*., 2001; Pradupsri *et al*., 2007) we predicted a role for RLIP76 in maturation and antigen internalization of DCs. This hypothesis was tested in present studies by examining cell surface expression of RLIP76 in monocyte-derived DCs, determining the effects of inhibiting RLIP76 functions on DC maturation and the effect of functional maturation arrest on DC-SIGN- and DEC-205-mediated receptor-ligand endocytosis. Flow cytometry was utilized to assess cell surface expression of activation markers and costimulatory molecules and confocal microscopy for studying colocalization of DC-specific endocytic CLRs and MHC-class II. The T cellstimulating capacity of DCs as a function of RLIP76 expression was determined in allo-MLR.

MATERIALS AND METHODS

Human subjects: Healthy volunteers gave informed, written consent and the Institutional Review Board of the University of North Texas Health Science Center approved these studies.

Cell culture medium: RPMI 1640 (Hy-Clone), supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), 10 mM HEPES (Sigma), 2 mM L-glutamine, 1 mM Na-Pyruvate (Sigma), 1x of MEM Non-essential amino acids (100x stock;

Sigma), 50 μM beta-mercaptoethanol and antibiotics (Penicillin G (Na salt)//Streptomycin sulfate, [100 U mL^{-1}//100 μg mL^{-1}], resp.) (GIBCO). This medium is further referred to as R-10.

Cytokines and growth factors: Human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) and human recombinant interleukin-4 (IL-4) were from R&D Systems (Minneapolis, MN). Bacterial lipopolysaccharide (LPS; *E. coli*, 0127:B8) was from Sigma-Aldrich (St. Louis, MO).

Antibodies: The anti-RLIP76 antibody was a polyclonal rabbit anti-human IgG purified in our laboratory (Singhal *et al*., 2003). Binding of this antibody, in flow cytometry or immune-cytochemistry experiments, was visualized by FITC-labeled goat anti-rabbit IgG. All other antibodies were fluorochrome-conjugated murine monoclonal antibodies from Becton Dickinson (BD, San Jose, CA).

Generation and cultures of DCs: Peripheral blood from healthy volunteers was obtained by phlebotomy into sterile, K2EDTA-containing Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) and mononuclear cells (PBMCs) were purified by Ficoll-Paque Plus (GE Healthcare Bio-science AB, Uppsala, Sweden) density gradient centrifugation as we described earlier (Zou *et al*., 2000). PBMCs were adhered to 6- or 12-well plates (Costar, Corning, NY) (107cells $3mL^{-1}$ or 5×106 cells/2 mL, resp.) for 2 h at 37o C in a humidified CO2 (5%) incubator in R-10. Non-adherent cells were gently rinsed away 3 times with RPMI 1640 (37°C) and DCs were generated from the adherent monocyte-enriched PBMCs in R-10 containing 100 ng of human GM-CSF mL^{-1} and 5 ng of human IL-4 mL^{-1} (cR-10). Non-adherent PBMCs were cryopreserved and used as responder cells in allo-MLR. Cell morphology was monitored daily under light microscope and flow cytometry was used for phenotyping on day 6 and 8. Fresh cytokines were supplemented every 2 or 3 days. Mature DCs (mDCs) were obtained by adding 1 μg LPS (*E. coli*)/mL on day 6 and culturing for 2 additional days. In antibody treatment experiments, day 5 iDCs were treated with anti-RLIP76 antibody or pre-immune IgG (PIS) (2.5 μg mL^{-1}, each) and were replenished on day 6. For confocal microscopy, antibody treatment started on day one and DCs treated with only LPS served as additional controls.

Morphology and immunofluorescence staining: 20,000 cells were cytospun, air-dried and, stained with modified Wright-Giemsa reagent (Sigma-Aldrich, St. Louis, MO) according to manufacturer recommended procedure. For immunofluorescence, cytocentrifuged cells were permeabilized/fixed with cold (4°C) methanol/acetic acid (3:1; V/V) for 5 min and washed 2-3 times with cold PBS. Non-specific interactions were minimized by pre-treating the cells with 10% goat serum in PBS for 60 min at room temperature. Cells were incubated with primary (anti-LIP76) antibody overnight at 4°C in a humidified chamber. Unbound antibodies were rinsed off with PBS (10 times, 3 minutes, each), cells were incubated for 1 h with FITC-conjugated goat anti-rabbit IgG at room temperature in a humidified chamber in dark. Unbound secondary antibody was removed by washing as before and cover-slips were mounted on slides with Vectashield mounting medium (Vector Laboratories). All operations beginning with addition of secondary antibody were carried out under reduced light. Rabbit PIS was used as negative control. Images were captured with Olympus AX70 fluorescence microscope.

Flow cytometry: DCs were stained on day 6 (iDCs) and day 8 (mDCs), unless otherwise stated. Cells were washed twice in cold staining buffer (Ca2+- and Mg2+-free PBS containing 2% heat-inactivated fetal calf serum and 0.09% sodium azide) and stained for 30 min at 4°C in 50 μL suspensions with the manufacturer recommended antibody dilution and 4 cell number. Following two washes with staining buffer, data were acquired on LSR II flow cytometer (BD, Mountain View, Calif.) and analyzed with FlowJo software (TreeStar, version 7.2.5.). DCs were never fixed or stored for more than 1 h before analysis. Positive cells were gated according to appropriate isotype controls and dot plots and histograms were generated for the monitored markers. Dead cells were excluded based on light scatter characteristics.

Mixed leukocyte reaction: Non-adherent PBMCs were incubated with graded numbers of DCs in 100-μL R-10/well in 96-well flatbottomed plates in triplicates. The anti-RLIP76 antibody or PIS were added at a final concentration of 2.5μg mL^{-1} and maintained throughout the culture period. To quantify T-lymphocyte proliferation, 20μL of CellTiter96 Aqueous One Solution Cell Proliferation Assay reagent (Promega) was added to cultures during the final 3.5 h of a 5-dayincubation period. Proliferation was assessed by reading the absorbance at 490 nm.

Endocytosis and laser scanning confocal microscopy: APC-conjugated anti-HLA-DR, Alexa Fluor 647-conjugated anti-CD205 (DEC-205), FITC-conjugated anti-CD209 (DC-SIGN) and DAPI (for Nuclear DNA) were used for staining. Cells were washed twice with cold staining buffer (as for flow cytometry), pre-cooled on ice for 30 min and stained for surface expression of HLADR, DEC-205 and DC-SIGN for 30 min at 4°C. Since, in our flow cytometry experiments, binding of nonspecific isotype control antibodies was always negligible, staining with these antibodies was omitted. Following two washes, cells were quickly re-suspended in 200 μL of R-10 and divided equally into two sterile tubes. One series was incubated at 37°C in CO2-incubator for 50 min to allow endocytosis to occur. The other series was kept on ice

for the same time (steady state control). Cells were then quickly cooled on ice, washed in cold staining buffer and fixed with 300 µL of 4% paraformaldehyde on ice for 1 h. After two washes, cells were cytospun and air-dried for 1 h. Cover slips were mounted with DAPI-containing Vectashield mounting medium and images of at least 10 randomly selected areas were captured on LSM 510 META confocal microscope (Zeiss) at 40x magnification. One out of the ten fields was captured at high pixel density for detailed imaging of individual cells. Images were analyzed using the Zeiss LSM Image Browser software (version R3.2) and mean fluorescence intensities per unit area were calculated using the ImageJ software (version 1.44 day).

RESULTS

RLIP76 is localized both in cytoplasm and on cell surface of monocyte-derived DCs: DCs were generated in the presence of GM-CSF and IL-4. Detection of RLIP76 on the cell surface by flow cytometry (Fig. 1) confirmed the presence of this protein in the cell membrane with at least an epitope on the surface. This is consistent with the known functions of RLIP76 in cytoplasm and in the cell membrane.

RLIP76 is a pre-requisite for maturation of monocyte-derived DCs: Maturation of either anti-RLIP76 antibody- or PIS-treated DCs was initiated on day 6 of culture. Antibodytreated or control day 6 iDCs and day 8 mDCs were analyzed for morphology and RLIP76 localization. The classical morphological differences between iDCs and mDCs are documented in Fig. 2 (panels A and B, resp). While only small processes are visible on the surface of iDCs, numerous long dendrites make the mDCs exceptionally well equipped for encountering other cells. Addition of anti-RLIP76 antibody to DCs 24 h before initiation of maturation inhibited the acquisition of fully mature morphology and "arrested" the cells in their immature state. This is indicated by morphological similarities between iDCs and the antibody-treated DCs on day 8 (Fig. 2A and D, resp.). On day 8, untreated or PIS-treated mDCs showed robust presence of long dendrites as an index of full maturity (Fig. 2B and C, resp.). Dendrites were shorter and much less developed in antibody-treated DCs (Fig. 2D) as compared to either untreated (Fig. 2B) or PIS-treated (Fig. 2C) DCs suggesting that functional RLIP76 was required during differentiation and maturation stages of DCs.

Maturation arrest of DCs by anti-RLIP76 antibody was also confirmed by flow cytometry. Although there is considerable variance in the percentage of RLIP76+ cells in iDC population (range: 0.3-31.8 %; n=25 and Fig. 1), These results validate the expression of RLIP76 on the cell surface. Even after subsequent delivery of the usual strong maturation booster, the bacterial LPS, antibody-treated DCs clearly showed an immature phenotype that expressed low levels of typical activation markers and co-stimulatory molecules, including CD83, HLA-DR,

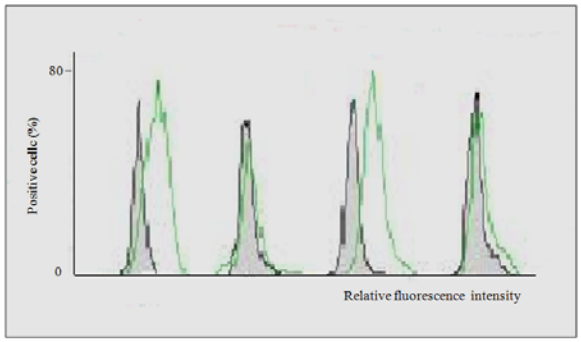

Fig. 1: Analysis of cell surface expression of RLIP76 by flow cytometry on human monocyte-derived immature dendritic cells. Day 6 iDCs of four randomly selected healthy volunteers (out of 25) were stained with 10 µg mL^{-1} anti-RLIP76 polyclonal antibody followed by FITC-conjugated goat anti-rabbit IgG and analyzed by flow cytometry. Filled histograms represent matching isotype controls, open histograms correspond to RLIP76+ cells. Varying levels of cell surface expression of RLIP76 was detected on iDCs (range: 0.3-31.8%; N=25).

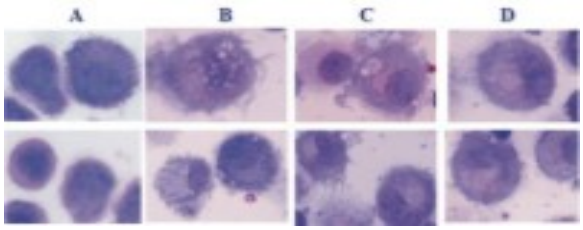

Fig. 2: Morphological effect of anti-RLIP76 antibody on maturation of monocyte-derived DCs. 2x104 cells were cytocentrifuged, air dried and stained with modified Wright-Giemsa staining (Sigma-Aldrich, St. Louis, MO) according to the manufacturer recommended procedure. In panel a, day 6 immature DCs are shown. Panels B, C and D show day 8 mature DCs generated by LPS in (B) absence, or presence of either (D) anti-RLIP76 antibody or (C) pre-immune serum (PIS). In each panel, two different fields are shown at 100×. Note the striking immature morphology of the specific anti-RLIP76 antibody treated DCs (panel D)

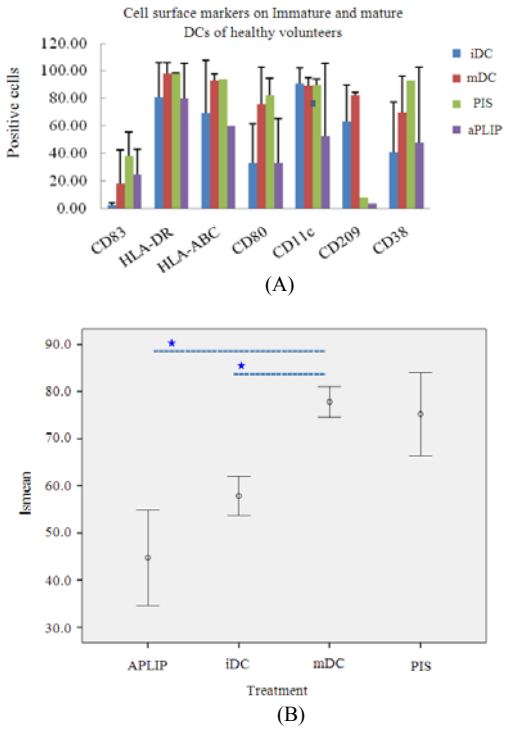

Fig. 3: Anti-RLIP76 antibody inhibits DC maturation. (A) For direct fluorescence, DCs (5x10$_4$) on day 6 (iDCs) or day 8 (mDCs) were washed twice in cold staining buffer and stained for 30 minutes at 4 $_o$C in 50 µL suspensions with a cocktail of fluorochrome-conjugated monoclonal antibodies according to the manufacturer recommended antibody dilution. Following two washes, flow cytometry data were acquired on LSR II flow cytometer (BD, Mountain View, Calif.) and analyzed with FlowJo software (TreeStar, version 7.2.5.). Expression of maturation markers and costimulatory molecules was markedly reduced when anti-RLIP76 antibody (aRLIP) was added to the DC culture 24 h before maturation initiation with LPS. For each marker, at least three independent analyses were performed. Means+SEM are shown. (B) Consequences of different treatments of dendritic cells, with respect to surface marker expression, were analyzed by statistical General Linear Model (GLM) procedure. Least square means of cumulative percentages of positive cells (LSMEAN) + SEM are plotted against the individual treatment groups (ARLIP: anti-RLIP76 antibody; iDC: day 6 iDCs; mDC: day 8 mDCs; PIS: Pre-immune serum IgG). Indicates significant differences with $p<0.02$ at 95% confidence limits. A total of 188 observations were read and used in our GLM procedure

HLAABC, CD80 and CD38, as compared to their untreated or PIS-treated counterparts (Fig. 3A). Together, these results confirmed the requirement of RLIP76 for maturation of DCs. When least square means of cumulative percentages of positive cells, expressing particular activation markers, were analyzed by statistical General Linear Model (GLM) procedure, significant differences ($p<0.02$; n=188) were obtained in simultaneous 95% confidence limits between iDCs and mDCs, as well as between anti-RLIP76 antibody pre-treated and untreated mDCs (Fig. 3B). However, there was no significant difference between iDCs and the antibody pre-treated "mDCs" ($p = 0.6072$) indicating that RLIP76 is critical for DC activation.

A total of 188 observations were analyzed during these studies, engendering high confidence in our morphological, phenotypical and functional observations implicating RLIP76 as an important factor required for unperturbed DC activation/maturation and for fulfilling the key role of DCs in shaping the adaptive immune response. Our findings provide compelling evidence for a novel and critical role for RLIP76 in differentiation and antigen internalizing functions of DCs. In addition, augmented presence of RLIP76 in DCs might infer an improved adaptive immune response through enhanced immune potentiating capacity of DCs. It is also possible that observed differences in RLIP76 expression in DCs contribute to inter-individual immune response variations.

Inhibition of allo-stimulatory function of DCs by anti-RLIP76 antibody: We next examined the immune stimulatory function of untreated and PIS-treated mDCs in allo-MLR andcompared it with that of anti-RLIP76 antibody-treated DCs. As shown (Fig. 2 and 3), antibody-treated DCs lack full maturation at day 8. When cryo-preserved, monocyte-depleted PBMCs (105) were co-cultured with graded numbers of differently treated DCs, the antibody pre-treated ones failed to fully stimulate allo-MLR as compared to their untreated or PIS-treated counterparts (Fig. 4). This indicates an indispensible role of RLIP76 in stimulatory function of DCs.

Clathrin-dependent endocytosis of DC-specific C-type lectin receptors (CLRs) is dramatically affected by anti-RLIP76 antibody: To investigate the role of RLIP76 in receptor-ligand endocytosis in DCs; we followed internalization of DCspecific CLRs, DC-SIGN and DEC-205. Co-localization of these receptors with each other, as well as with MHCclass II was assessed by comparing Pearson correlation coefficients of co-localization. iDCs and mDCs were compared at both steady-state (4°C) and endocytic (37°C) conditions and untreated or rabbit pre-mmune Ig Gtreated DCs served as controls.

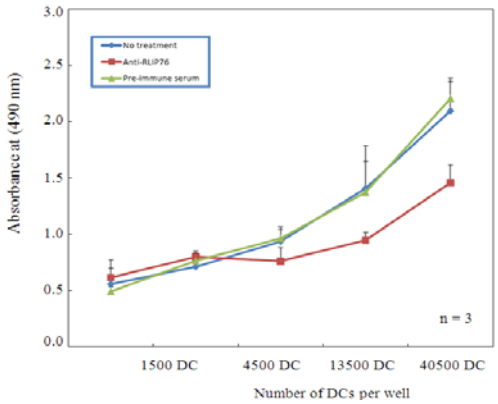

Fig. 4: Effect of anti-RLIP76 antibody treatment on DC-induced allo-MLR. Non-adherent PBMCs were incubated with graded numbers of mDCs in 100-µL of R-10/well in 96-well flat-bottomed plates in triplicates. Anti-RLIP76 antibody or pre-immune IgG were added to the similarly pre-treated cells at a final concentration of 2.5µg/mL. To quantify T-lymphocyte proliferation, 20µL of CellTiter96 Aqueous One Solution Cell Proliferation Assay reagent (Promega) was added to cultures during the final 3.5 h of a 5-day incubation period and proliferation was assessed by reading the absorbance at 490 nm. Pre-treatment of DCs with anti-RLIP76 antibody reulted in their impaired induction of T cell proliferation in allo-MLR. One representative experiment out of three with similar results is shown. Each data point represents an average of triplicates+SEM

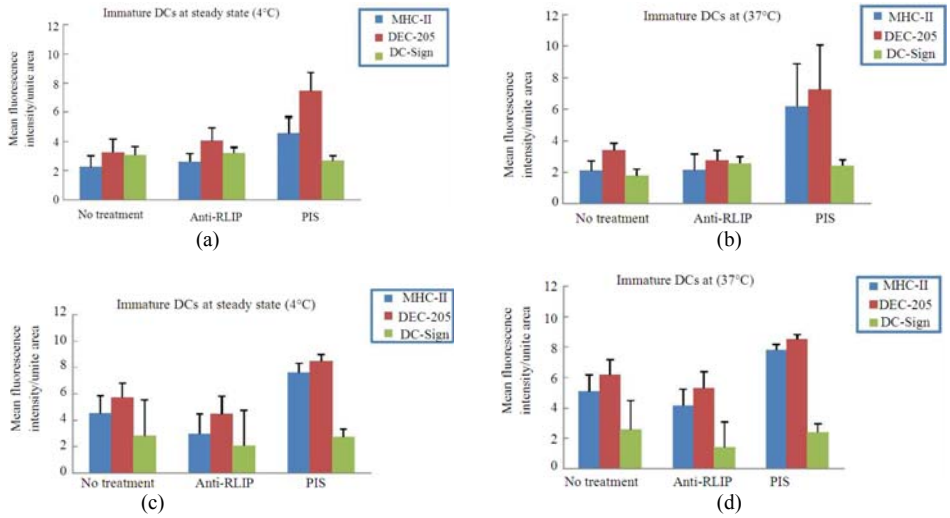

Fig. 5: Mean fluorescence of differently treated immature and mature DCs at steady state (4°C) and endocytic (37°C) conditions. DCs were cultured in cR-10 and maturation was initiated with LPS on day 6. Anti-RLIP76 antibody or PIS was added to the cultures on day one (2.5 µg mL^{-1}) and replenished every other day together with the cytokines. For endocytosis studies, cells were washed 2 times in staining buffer (s.b.), pre-cooled on ice for 30 min and stained for surface expression of MHC-class II, DEC-205 and DC-SIGN. Following two washes in s.b., cells were resuspended in 200 µL of cold R-10 medium and equally distributed into two tubes. One series of differently treated cells was incubated at 37°C in a humidified CO2-incubator for 50 minutes (endocytic condition), while the other series was kept on ice for the same time (steady state condition). Following incubation, cells were quickly cooled, washed in cold s.b. and fixed with 4% paraformaldehyde on ice. After two washes, cells were cytospun, air-dried for 1 hour and cover slips were mounted with DAPI-containing Vectashield mounting medium. High pixel density images were captured on LSM 510 META confocal microscope (Zeiss) at 40x magnification. Images have been analyzed using the Zeiss LSM Image Browser software (version R3.2) and the ImageJ (version 1.44d). Average fluorescence intensities normalized per unit area of 10 different fields + SEM are shown

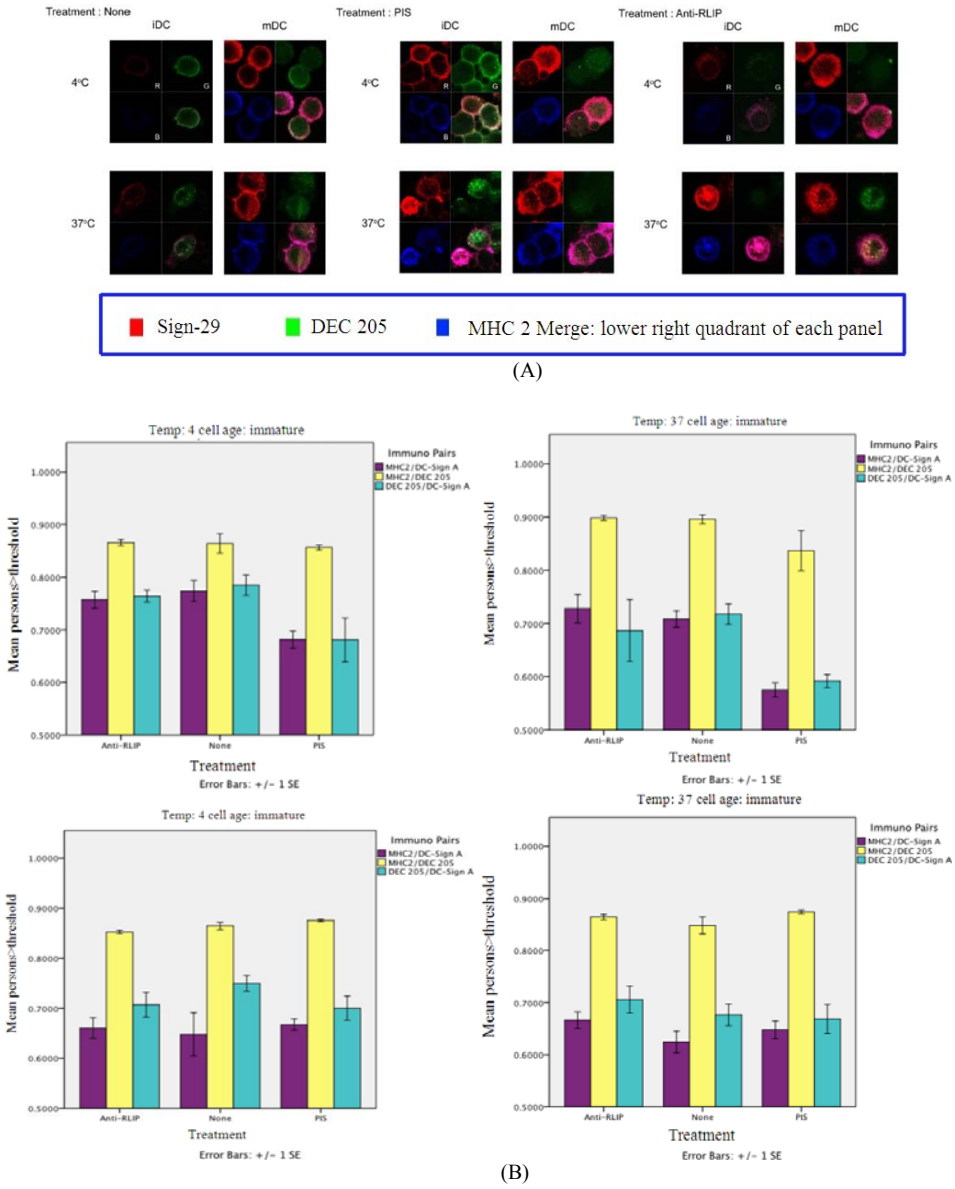

Fig. 6: Endocytosis and colocalization of MHC-class II, DC-SIGN and DEC-205 by differently treated iDCs and mDCs as assessed by confocal microscopy. (A) Detailed snap-shots of iDCs and mDCs at steady state (4 $_{o}$C) and at the end of a 50-minute endocytic (37 $_{o}$C) condition are shown. DCs were cultured in cR-10 and maturation was initiated with LPS on day 6. Anti-RLIP76 antibody or PIS was added to the cultures on day one (2.5 μg mL^{-1}) and replenished every other day. High pixel density images were captured on LSM 510 META confocal microscope (Zeiss) at 40x magnification. Images were equally zoomed for detailed view of cellular structures and analyzed using the Zeiss LSM Image Browser software (version R3.2). Individual markers are color-coded as indicated in the legend. DAPI was used for nuclear DNA staining (not shown). Merged images for individual treatment conditions are shown in the lower right quadrants of each panel. (B) Mean Pearson correlation coefficients of colocalization normalized for the background fluorescence are plotted against treatment conditions. For statistical evaluation, fluorescence intensities of at lease eight different fields for each treatment condition were included. Colocalization is color-coded as indicated in the legend. Clearly, colocalization of MHC-class II and DEC-205 is far superior to colocalization of any other receptor pairs at any tested conditions. Shown values are averages + 1SEM.

As no natural ligands for DEC-205 have been identified as of today, fluorochromeconjugated monoclonal antibodies served as natural ligand surrogates for all the receptors. Mean fluorescence intensities of cells captured in ten different fields for each treatment condition are normalized per unit area and are plotted in Fig. 5. Comparing these figures with detailed images of individual cells (Fig. 6A) we discern that the major function of iDCs is antigen internalization, they heavily rely on both macropinocytosis and CDE and, at steady state (4°C) condition, little fluorescence is apparent at either plasma membrane or inside the cells with the exception of the PIS-treated iDCs. This overall picture has not substantially changed even at the end of the 50 min endocytic (37°C) cycle, with the exception of a slight DC-SIGN fluorescence increase in the anti-RLIP76 antibody-treated cells compared to untreated or PIS-treated ones. A good deal of DC-SIGN has been internalized and is co-localized in perinuclear area with MHC-class II, although a fair amount still remained in the cell membrane.

Rabbit IgG stimulated the endocytosis of all three receptors, most prominently, that of DEC-205 and MHC-class II. It exhibited strong maturation effect on human, monocyte-derived DCs and considerably enhanced the rate of CDE. This observation is consistent with the reported enhanced antigen-presenting capacity of DCs (up to 100 times) due to rapid internalization and recycling of both DEC-205 and antigen-complexed IgG through FcγRI (CD64) and FcγRII (CD32) (Larsson *et al*., 1997; Renault *et al*., 1999; Jiang *et al*., 1995). While at steady state the prevailing proportion of all three receptors was clearly localized at the plasma membrane with some diffuse-most probably degraded-cytoplasmic DEC-205 (Fig. 6A), at the end of the 50 min endocytic cycle all were tightly packed in the perinuclear region with some remainder DC-SIGN and MHS-class II still on the cell surface.

mDCs exhibited far more dramatic receptor-ligand trafficking than iDCs, both on cell membrane and intracellularly. This is reflected by an overall increase in mean fluorescence intensities, except for that of DC-SIGN in anti-RLIP76 antibody-treated DCs at endocytic and for all receptors at steady state conditions (Fig. 5B). Thus, our results show functional involvement of RLIP76 in antigen internalization and presentation by mDCs that can be profoundly suppressed by specific anti-RLIP76 antibodies. Although maturation of DCs has long been assumed to be accompanied by down-regulation of endocytosis, recent studies provide evidence that despite their downregulated capacity for non-specific macropinocytosis, mDCs continue to form clathrin-coated vesicles (Garrett *et al*., 2000) and to accumulate antigens by receptor-mediated endocytosis and phagocytosis (Platt *et al*., 2010; Gil-Torregrosa *et al*., 2004; Shi *et al*., 2008). While DC-SIGN and MHC-class II, at steady state, appeared on the cell surface of untreated mDCs in flourishing, sharp coronas, DEC-205 appeared abundantly also in the cytoplasm (Fig. 6A, left). This latter pattern was even more pronounced in the PIS-treated mDCs with only remnants of DEC-205 in the cytoplasm and none on the plasma membrane (Fig. 6A, middle). In antibody-treated mDCs, all DEC-205 appeared as a diffuse background inside the cells (Fig. 6A, right). While, at a first glimpse, no apparent difference in surface abundance of DC-SIGN and MHC-class II might be obvious between PIS- and antibody-treated mDCs, a more thorough look reveals much thicker and luxuriant presence of these receptors on the PIS-treated cells. This, again, is completely in line with published data (Larsson *et al*., 1997; Renault *et al*., 1999; Mahnke *et al*., 2000).

The most revealing effect of anti-RLIP76 antibody treatment on endocytosis and antigen trafficking was apparent on mDCs that have undergone the 50 min endocytic cycle. While faint clusters of both CLRs and MHCclass II were still visible in the cytoplasm of untreated mDCs, they almost completely dispersed in the PIS-treated ones (Fig. 6A, lower panels). DC-SIGN and MHC-class II were greatly depleted from the cell membrane, although the surface of PIS-treated mDCs had still more of them compared to the untreated ones. On the other hand, these receptors appeared in higher amounts in the cytoplasm of untreated mDCs than in the PIS-treated ones. This apparently indicates efficient recycling and much higher turn-over rate of endocytic machinery in the latter. All receptors in antibody-treated mDCs appeared in tight layers near the plasma membrane with abundant clusters in the perinuclear area. This rather slow progress of receptor-mediated internalization of surrogate legends clearly reflects the inhibitory effect of anti-RLIP76 antibody on DC differentiation/maturation and uplohds our observation on impaired antigen-presenting efficiency of these cells in allo-MLR assay (Fig. 4).

To further confirm our observations, co-localization of CLRs and MHC-class II was studied in differently treated mDCs and iDCs. Plots of mean Pearson correlation coefficients of co-localization are shown in Fig. 6B. Colocalization of MHC-class II and DEC-205 was found to be far superior, under all conditions, to co-localization of any other receptor pairs (Table 1). Fairly close co-localization of MHC-class II/DC-SIGN and DEC-205/DC-SIGN was also apparent for untreated or antibody-treated steady state iDCs (Fig. 6B, upper left panel).

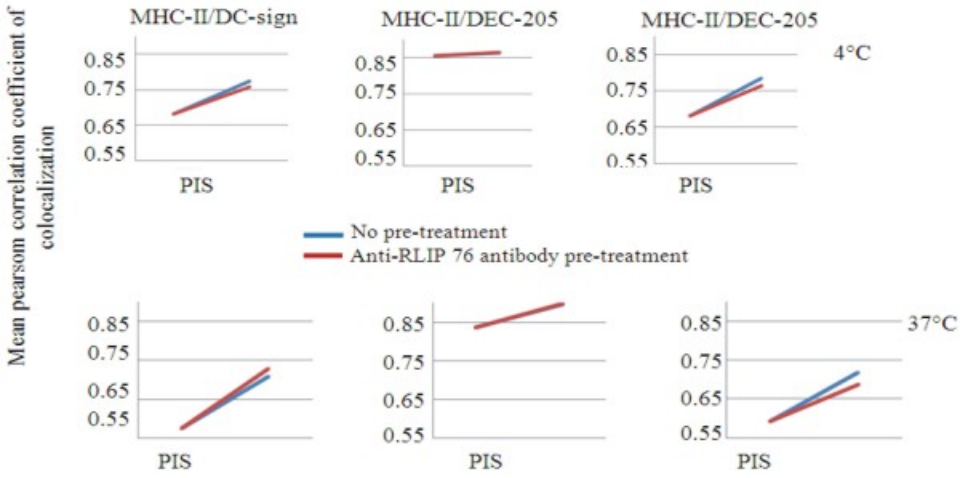

Fig. 7: Only iDCs exhibit significant differences in Pearson correlation coefficients of colocalization for different treatment conditions and endocytic receptor pairs. Trends of changes in mean Pearson correlation coefficients for DCs are shown. DCs were evaluated in either steady state (4°C) or endocytic (37°C) condition. Pre-treatments are color-coded as indicated in the legend. Each comparison referres to PIS pre-treatment, as values of correlation coefficients appeared to be the lowest ones for this condition. For each condition, data represent means of at least 8 independent evaluations and with $p<0.02$ (T-test) for both MHC-II/DC-SIGN and DC-SIGN/DEC-205

Table 1: Mean Pearson Correlation Coefficients of Colocalizations. A total of at least 8 data point for each treatment condition has been used for statistical evaluation

	Pretreatment	MHC-II/DCSIGN	MHCII/DEC-205	DCSIGN/DEC-205
iDC 4oC	None	0.7736	0.8641	0.7847
	anti-RLIP	0.7571	0.8658	0.7641
	PIS	0.6813	0.8561	0.6809
37°C	None	0.7083	0.8958	0.7176
	anti-RLIP	0.7280	0.8984	0.6866
	PIS	0.5750	0.8368	0.5919
mDC 4°C	None	0.6478	0.8647	0.7497
	anti-RLIP	0.6603	0.8525	0.7068
	PIS	0.6673	0.8757	0.7000
37°C	None	0.6242	0.8480	0.6763
	anti-RLIP	0.6662	0.8644	0.7058
	PIS	0.6472	0.8740	0.6683

When differences in Pearson correlation coefficients of co-localization of individual CLRs and MHC-class II molecules-as a function of DC maturation stage, pre-treatment, or endocytic *versus* steady state conditions-were examined, only iDCs were found to exhibit such differences (Fig. 7 and Table 1). At both steady state and endocytic conditions, significant differences in co-localizations were found between PIS-treated and either untreated or antibody-treated DCs ($n=8$; $p<0.02$, T-test). However, there was no significant difference in co-localization of MHC-class II and DEC-205 with respect to pre-treatment, since these co-localized with high correlation under all conditions (Fig. 7, middle plots). Likewise, no significant differences were found between untreated and RLIP76- specific antibody-treated iDCs under either steady state or endocytic conditions. Clearly, the observed differences in receptor co-localizations in iDCs were inferred by the maturation effect of rabbit IgG, while the MHC-class II/DEC 205 pair co-localized with high correlation under all conditions.

DISCUSSION

In present studies, we generated and studied human dendritic cells from the blood of healthy volunteers and used immuno-cytochemistry, flow cytometry and functional assays to demonstrate a critical requirement of RLIP76 for functional maturation of DC. Our data confirm the predictions of our previous studies showing

an absolute requirement of RLIP76 for CDE and are consistent with multiple studies by others which indicate the requirement of CDE for functional maturation of dendritic cells (Garrett *et al*., 2000; Platt *et al*., 2010; Kobayashi *et al*., 2001).

Immature DCs can endocytose vigorously through a variety of mechanisms including nonspecific uptake by constitutive macropinocytosis and phagocytosis and specific uptake via CDE and CvDE. Although the hallmark of maturation is down-regulation of endocytosis which is widely assumed to restrict the ability of mDCs to capture and present antigens, Platt *et al*. have recently reported that mDCs continued to form clathrin-coated vesicles and accumulated antigens, especially by receptor-mediated endocytosis and phagocytosis (Platt *et al*., 2010). FcγR-mediated delivery of immune-complexes (ICs) to DCs was shown to result in efficient peptide loading of MHC-class II molecules and to promote MHC-class I-restricted cross-presentation at very low antigen concentration (Renault *et al*., 1999; Jiang *et al*., 1995; Amigorena and Bonnerot, 1999)]. Mahnke *et al*. (2000) reported a new pathway for DEC-205 cytosolic domain which mediated receptor-ligand endocytosis and entailed efficient recycling through late endosomes and greatly enhanced efficiency of antigen presentation to CD4+ T cells. DEC-205 has also been shown to be far superior to the Macrophage Mannose Receptor (MMR) in presenting bound rabbit antireceptor antibodies to T cells, a classical assay for measuring the presenting function of endocytic receptors (Chesnut and Grey, 1981).

RLIP76, the first effector of Ral, is a major determinant of the rate of receptor-ligand complex initiated signaling, the subsequent clathrin-coated pit mediated endocytosis and termination of signaling (y). The ATPase activity of RLIP76 is the key player in this process, possibly as an energy transducer, that is coupled with efflux of endogenous substrates (allocrites), particularly GS-E. In our recent communication (Awasthi *et al*., 2010), this has been confirmed in *RLIP*-/- mice that are deficient in CDE. In these studies, RLIP76 was induced by oxidative or hyperglycemic stress and theconcomitant increase in insulin endocytosis was completely abrogated by inhibiting transport activity of RLIP76. Recently, involvement of at least two GTPases - Cdc42 and Rac1- in signaling cascades inducing phenotypic and functional maturation of human DCs has been demonstrated (y). As we previously reported (Singhal *et al*., 2009a), cdc2, a cell cycle check point control kinase, binds to RLIP76 and its increased expression inhibits transport function of RLIP76 as well as endocytosis. Phagocytosis, as well as antigen uptake through the nonspecific mechanism of constitutive macropinocytosis is markedly down-regulated during DC maturation, attributable, partly, to a decrease in active form of the Rho GTPase, Cdc42. It is, therefore, well conceivable that inhibiting the GTPase Activating Protein (GAP) function of RLIP76 in DCs during their *in vitro* differentiation will down-regulate their endocytic activity owing-at least partly-to decreased presence of active forms of Cdc42 or other Rho GTPases. It would also be interesting to pursue whether suppressing functional RLIP76 in DCs would, in turn, increase the expression of cdc2. In our present study we wondered what effect-if any-inhibition of functional RLIP76 in human DCs would have on internalization of the DC-specific endocytic CLRs, DC-SIGN and DEC-205 and the antigen presenting molecule, MHC-class II.

The design of present studies included DCs pre-treated with either RLIP76 specific antibody or pre-immune rabbit IgG, along with the untreated controls. Maturation of treated and untreated DCs was initiated by bacterial LPS on day 6 and continued for two additional days. The progress to terminal maturation was examined by several criteria. In addition to morphological changes, DC maturation was monitored by flow cytometry checking cell surface expression of common myeloid DC markers, activation markers, co-stimulatory and adhesion molecules. Immuno-cytochemical analysis combined with flow cytometry revealed the location of RLIP76 in the cytoplasm as well as on the cell surface of DCs. The expression profiles of RLIP76 in iDCs exhibited considerably high variance(range: 0.3-31.8%; n=25 and Fig. 1) that was similar to the reported differential expression of other transporters, including various ABC family transporters or Solute Carrier Organic Anion Transporters (SLCO) (Skazik *et al*., 2008; Laupèze *et al*., 2001), which play important roles in DC differentiation, homing and migration (Robbiani *et al*., 2000; Block and Markovic, 2009). Anti-RLIP76 antibody-treated DCs clearly showed maturation arrest as judged by all criteria of maturation used in this study. While PIS-treated DCs reached activation levels comparable with those of untreated mDCs, antibody-treated DCs remained closely similar to iDCs (Fig. 2 and 3). The statistical significance of these differences, as judged by the GLM procedure (Fig. 3B), further corroborates the importance of functional RLIP76 for proper DC maturation. The antibody-treated DCs were also impaired in their T cell stimulatory potency in allo-MLR, as compared to their untreated or PIS-treated counterparts (Fig. 4). These findings indicate that, similar to Cdc42 and Rac1, RLIP76 plays an important

role in DC activation/maturation and that anti-RLIP76 antibodies inhibit this process. Our results also provide evidence for the requirement of this non-ABC multifunctional transporter in cellular mechanisms that underlay membrane plasticity and trafficking and are consistent with some of the observed functional similarities between RLIP76 and the ABC transporters. As demonstrated, confocal microscopy yielded similar or much weaker fluorescence intensities for all receptors in RLIP-specific antibody-treated DCs, compared to untreated or PIS-treated cells (Fig. 5). This clearly reflects the inhibitory effect of RLIP76-specific antibody on DC membrane trafficking and reinforces our observations of impaired antigen-presenting capacity of these cells in MLR, as well as the pronounced deregulation of DC maturation markers and co-stimulatory molecules detected by flow cytometry. Furthermore, these observations support our finding on DC-maturing effect of rabbit PIS which up-regulated most of the above mentioned markers compared to the antibody-treated DCs. The mechanistic basis for this role of RLIP76 needs to be investigated.

Recently, many new PRRs have been identified on DCs that may contribute to their function at the interface of innate and adaptive immunity [e.g., CLRs, Toll-Like Receptors (TLRs), NOD-Like Receptors (NLRs), DCimmunoreceptor (DCIR)]. While TLRs are specialized mainly for recognizing both extracellular and intracellular microbial agents in the milieu of inflammatory signals, including proteins, lipoproteins, viral-, bacterialas well as damaged self- RNA/DNA, CLRs serve primarily as antigen receptors recognizing pathogens through their specific carbohydrate structures. It has become clear, however, that in the realm of highly diverse C-type lectins on DCs, some can regulate cell adhesion, migration, signaling, membrane trafficking and cytoskeletal processes (Engering *et al*., 2002; Platt *et al*., 2010; Mahnke *et al*., 2000; Geijtenbeek *et al*., 2002; Valera *et al*., 2008). Sensing and transducing signals from the outer cellular environment into subcellular compartments include constitutive, non-specific macropinocytosis, phagocytosis and receptor-mediated CDE and CvDE.

There is growing evidence that signaling pathways and endocytic pathways are regulated in a reciprocal manner and transcription is directly controlled by endocytic proteins. The Heavy Chain of Clathrin (CHC) itself can be found in the nucleus where it specifically enhances p53-dependent transactivation by binding to the p53-responsive promoter and stabilizing the interaction between p53 and the histone acetyltransferase p300. Given the different protein kinase and kinase phosphorylation motifs in the cytoplasmic tail of RLIP76 (Awasthi *et al*., 2002), its involvement in downstream signaling and induction of cytokine production is well conceivable. In addition, its GAP domain shows specificity for the Rho family members, Cdc42 and Rac1, both of which were found to be critical for monocyte derived DC polarization and motility, as well as for formation of long processes for DC adhesion to extracellular matrix-coated surfaces (Burns *et al*., 2001; Swetman *et al*., 2002). Inhibition of Rho GTPase activity in monocyte-derived DCs, on the other hand, induced the loss of the constitutive filamentous surface projections and reduced the alloantigen-presenting capacity of these cells (Kobayashi *et al*., 2001). These reports speak in favor of our results and support the critical role of RLIP76 in antigenpresenting and immune-stimulatory function of DCs. Cellular mechanisms that regulate the accompanying membrane trafficking, play crucial roles in DC maturation/activation. Anti-RLIP76 IgG, as we demonstrated here, Clearly, arrested" this process in monocyte-derived human DCs.

Adjuvant immunotherapy represents a strong adjunct arsenal to standard surgery, radiation- and chemotherapy of cancer and DC-based approaches constitute the leading immune enhancing strategy. The tumor environment provides tumor-associated antigens for immune stimulation as well as immune cells attempting to destroy the tumor. At the same time, however, tumor tissues contain high levels of cells with all the hallmarks of regulatory T (Treg) cells (CD4+ CD25+ Treg). These migrate into the tumor microenvironment in a process mediated by chemokine CCL22 and are capable of suppressing antitumor responses. Thereby, DCs present *in situ* in the tumor are maintained in an inactive (immature) state promoting immunological tolerance to tumor antigens (Sioud, 2009). Moreover, tumor antigens released by dying tumor cells following chemo-, cryo-, or radiation therapy are not efficiently crosspresented *in vivo* by DCs due to their dysfunction and/or limited number *in situ*. RLIP76 has been shown to be over expressed in a number of types of cancer and appears to be necessary for cancer cell survival. Our *in vivo* studies demonstrate that administration of anti-RLIP76 antibodies, siRNA or anti-sense DNA to mice bearing xenografts of PC-3 prostate cancer leads to near complete regression of tumor with no apparent toxic effects (Singhal *et al*., 2009b). Cancer cells also appear significantly more sensitive than normal cells to apoptosis triggered by blocking RLIP76 (Awasthi *et al*., 2003; Singhal *et al*., 2009a), suggesting the feasibility of targeting RLIP76 in human cancer therapy. It is tempting to speculate that RLIP76 mediated immune response-through its

involvement in maturation, antigen-internalizing, antigen-presenting and T cell-stimulating function of DCs - may be a determinant of differential sensitivities to anti-RLIP76 IgG of normal and cancer cells, the latter having markedly increased levels of RLIP76.

CONCLUSION

In summary, we have demonstrated for the first time a dramatic inhibitory effect of anti-RLIP76 antibody on the phenotype and allogeneic T cell-stimulatory function of monocyte-derived human dendritic cells *in vitro*. The observed differences in RLIP76 expression by DCs from healthy volunteers' PBMCs may contribute to existing inter-individual immune response variations. Clathrin-dependent endocytosis, through DC-specific CLRs, DC-SIGN and DEC-205, is shown to be substantially impaired in anti-RLIP76 IgG-pretreated DCs. Rabbit pre-immune IgG, on the other hand, considerably enhanced CDE in both immature and mature DCs under either steady state or endocytic condition. These findings imply that augmented presence of RLIP76 in DCs should contribute to improved adaptive immune response through enhanced immune-potentiating capacity of DCs and further studies are underway in our laboratory to explore this possibility.

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