Polysialic acid is required for neuropilin-2a/b-mediated control of CCL21-driven chemotaxis of mature dendritic cells and for their migration in vivo

Angela Rey-Gallardo2, Cristina Delgado-Martín2, Rita Gerardy-Schahn3, José L. Rodríguez-Fernández2, and Miguel A. Vega1,2

2Departamento de Microbiología Molecular y Biología de las Infecciones, Centro de Investigaciones Biológicas, CSIC, Madrid, Spain and
3Medizinische Hochschule Hannover, Institute für Zelluläre Chemie, Hannover, Germany

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Migration of mature dendritic cells (mDCs) to secondary lymphoid organs is required for the development of immunity. Recently, we reported that polysialic acid (PSA) and the transmembrane glycoprotein neuropilin-2 (NRP2) control mDC chemotaxis to CCL21 and that this process is dependent on the C-terminal basic region of the chemokine. Herein, we provide further insight into the molecular components controlling PSA regulated chemotaxis in mDCs. In the present study, we demonstrate that human mDCs express the NRP2 isoforms NRP2a and NRP2b, that both of them are susceptible to polysialylation and that polysialylation is required to specifically enhance chemotaxis toward CCL21 in mDCs. To investigate the relevance of polysialylation on mDC migration, a xenograft mouse model was used and the migration of human DCs to mouse lymph nodes analyzed. Here, we demonstrate that the depletion of PSA from mDCs results in a drastic reduction in the migration of the cells to draining popliteal lymph nodes. With this finding, we provide first evidence that PSA is a crucial factor for in vivo migration of mDCs to lymph nodes.

Keywords: dendritic cells / migration / neuropilin-2 / polysialic acid / ST8SiaIV

Introduction

Pathogen sensing by tissue dendritic cells (DCs) triggers a complex regulated genetic program that induces their maturation (Steinman and Banchereau 2007). Mature DCs (mDCs) acquire the capabilities to process and present the captured antigens and migrate to lymph nodes, where they activate antigen-specific T lymphocytes, thereby initiating the adaptive immune response against the infection (Alvarez et al. 2008). The migration of mDCs to lymph nodes is controlled by the CCR7-dependent activation of multiple G-protein-dependent signaling pathways, induced by the interaction between the CCR7 receptor and its chemokine ligands CCL21 and CCL19 (Randolph et al. 2008). Besides CCR7, accessory molecules, such as the ADP-ribosyl cyclase CD38 and the PGE2 receptors EP2 and EP4, affect mDC migration to secondary lymphoid organs, likely by regulating CCR7-associated signaling cascades (Randolph, Sanchez-Schmitz et al. 2005).

In independent studies, we and others have recently demonstrated that the presence of polysialic acid (PSA) 2,8-linked N-acetylmuraminic acid enhances the chemotaxis of monocyte-derived human mDCs toward CCL21 (Bax et al. 2009; Rey-Gallardo et al. 2010). Moreover, we found that chemotaxis toward CCL19 was not influenced by PSA (Rey-Gallardo et al. 2010). Consequently, the depletion of PSA form mDCs decreased CCL21- but not CCL19-mediated activation of the JNK and Akt signaling pathways, both of them associated with CCR7-dependent migration (Rey-Gallardo et al. 2010). Importantly, the enhancing effect of PSA on CCL21-driven chemotaxis was shown to depend on the highly basic C-terminal region present in CCL21 (Rey-Gallardo et al. 2010).

The cell surface expressed glycoprotein neuropilin-2 (NRP2) acts as a receptor for some members of the VEGF family and for a number of class 3 semaphorins (Pellet-Manly et al. 2008). Thereby, NRP2 associates with VEGF receptors upon VEGF binding and enhances VEGF receptor activity and signaling (Geretti et al. 2008). Similarly, NRP2 acts as co-receptors for plexins and transmembrane receptors that activate signal transduction pathways upon SEMA3F binding (Shimizu et al. 2008). The interactions of NRP2 expressed in endothelial cells and some tumors with VEGF/SEMA3 are thought to be responsible of the assigned NRP2 roles in angiogenesis, cell migration and tumor progression (Bielenberg et al. 2006; Geretti et al. 2008; Pellet-Manly et al. 2008).
NRP2 expression is induced during DC maturation, and it is known to carry PSA attached to O-linked glycans (Curreli et al. 2007). We have previously demonstrated that the siRNA-mediated knocking down of NRP2 in DCs, selectively decreases their chemotaxis toward CCL21 and thus identified NRP2 as a regulator of mDC migration (Rey-Gallardo et al. 2010).

To gain a deeper understanding of the molecular mechanisms that underlie the PSA enhancement of CCL21-driven mDC migration, we first investigated the overall expression of NRP2 and its isoforms in mDCs. Our data indicate that most mDCs expressing PSA also expressed NRP2 and that NRP2 appears in mDCs as two isoforms (NRP2a and NRP2b). We also demonstrate that both forms are the targets of polysialylation in mDCs and that their polysialylation is required for CCL21-stimulated mDC migration. Finally, in a xenograft mouse model, the migration of extra corporally human DCs to draining popliteal lymph nodes was visualized after subcutaneous injection into mouse footpads. This in vivo model clearly demonstrated that PSA on mDCs is essential for the efficient migration of these cells to secondary lymphoid organs.

**Results**

*Human mDCs express the NRP2 isoforms, NRP2a and NRP2b, and both are the targets of the polysialyltransferase ST8SiaIV*

To investigate the overall expression of polysialylated NRP2 on mDCs, we carried out flow cytometry analysis of mDCs stained with both anti-NRP2 and anti-PSA antibodies. As shown in Figure 1A, most of the PSA + DC population is also positive for NRP2 expression. This observation, which is consistent with previously published western blot analysis on PSA expression in mDCs (Curreli et al. 2007; Rey-Gallardo et al. 2010), strongly suggests that NRP2 is the most abundant polysialylated protein present on mDCs. Interestingly, the existence of a minor PSA + NRP2− DC population indicates that besides NRP2, other polysialylated proteins must be present on mDCs.

The NRP2 gene encodes several mRNA isoforms (Rossignol et al. 2000). Among these, there are two major transmembrane isoforms, designated as NRP2a and NRP2b, differing only in their transmembrane and cytoplasmic domains. To identify the NRP2 isoforms present in mDCs, we set up a RT-PCR assay to analyze their relative expression levels in human LPS-induced DCs (Figure 1B). We used as amplification controls for NRP2 in mDCs, cDNAs derived from RNA isolated from the tumor cell lines A375 and HEK293, which showed by flow cytometry analysis comparable cell surface expression levels of NRP2 to mDCs (Supplementary data, Figure S1). Expression of NRP2a and NRP2b isoforms in mDCs generated from several independent donors revealed that both isoforms were consistently detected in mDCs (Figure 1B). However, the NRP2a/NRP2b expression ratio was not equivalent for all donors, suggesting a donor-dependency the regulation of the relative expression of both isoforms. In the absence of specific antibodies to distinguish the NRP2a/NRP2b isoforms, the small difference in size between both isoforms (NRP2a: 931 amino acids; NRP2b: 906 amino acids) precludes their identification at the protein level by western blot (data not shown). These results demonstrate that human mDCs express the mRNA corresponding to both the NRP2a and NRP2b isoforms.

NRP2b: 906 amino acids) precludes their identification at the protein level by western blot (data not shown). These results demonstrate that human mDCs express the mRNA corresponding to both the NRP2a and NRP2b isoforms. The involvement of NRP2 and PSA in the regulation of
CCL21-directed chemotaxis (Rey-Gallardo et al. 2010), and the fact that NRP2 can be found polysialylated in mDCs (Curreli et al. 2007; Rey-Gallardo et al. 2010), strongly suggest that regulation of mDC chemotaxis toward CCL21 requires polysialylated NRP2. To explore this assumption, we first investigated whether both NRP2 isoforms are targets of the ST8SiaIV, the only polysialyltransferase expressed in mDCs (Curreli et al. 2007). For this purpose, COS7 cells were co-transfected with expression vectors encoding for the ST8SiaIV and for either NRP2a or NRP2b. Twenty-four hours after transfection, one half of the cells were treated with EndoN and the second half were left untreated, and NRP2 protein expression was analyzed in cell lysates by western blotting. As shown in Figure 1C, treatment of both the NRP2a and NRP2b transfected cells with EndoN caused the disappearance of the smear band displayed with the NRP2 antibody and revealed a smaller in size discrete band. This change in mobility is a feature consistent with the removal of PSA from the carrier protein by EndoN. Identical results were obtained when human K562 cells were used (Supplementary data, Figure S2). Taker together, these results indicate that both NRP2a and NRP2b are targets of the polysialyltransferase ST8SiaIV.

NRP2a and NRP2b must be polysialylated to regulate CCL21-driven chemotaxis in mDCs

We next investigated whether both NRP2 isoforms are also polysialylated in mDCs and whether the control of mDC migration toward the CCL21 and CCL19 chemokines requires their polysialylation. Therefore, immature DCs were nucleofected with expression vectors encoding for NRP2a and NRP2b, respectively, or with an empty control vector. The cells were matured in the presence of LPS for 18 h, and then used for in vitro chemotaxis assays. An 18 h maturation time was chosen to limit as much as possible the up-regulation and polysialylation of endogenous NRP2, but allowing the detection of the recombinant nucleofected NRP2. In this respect, and as evidenced through investigation of the kinetics of expression of NRP2 along DC maturation, although NRP2 protein expression started to appear 12 h after the addition of the maturation stimulus, polysialylation was only observed later on (Figure 2A). By flow cytometry using anti-NRP2 and anti-PSA antibodies, mDCs transfected with either NRP2a or NRP2b were found to express higher NRP2 and PSA levels than mDCs transfected with a control plasmid (Figure 2B). Treatment with EndoN fully removed the PSA without altering the expression of NRP2 (Figure 2B). Identical results were obtained in the western blot analysis shown in Figure 2C. Cells transfected with NRP2 isoforms show stronger NRP2 signals before and after EndoN treatment of samples, and a wider NRP2 band smear (indicative of PSA content and observed in the EndoN-untreated samples) with respect to cells nucleofected with the control vector. In summary, these results strongly indicate that, as expected on the basis of the experiments carried out in COS7 cells, both NRP2a and NRP2b are polysialylated in mDCs.

Finally, to determine whether the polysialylation of NRP2a and NRP2b influences mDC chemotaxis, mDCs overexpressing the NRP2 isoforms were used in chemotaxis experiments. Overexpression of each of the two NRP2 isoforms resulted in an enhancement of the CCL21-driven chemotaxis. In contrast, no influence in the response to CCL19 was observed (Figure 2D). Interestingly, the removal of PSA by EndoN treatment of DC nucleofected with either the control vector or the NRP2a/NRP2b vectors reduced to the similar basal levels their chemotaxis toward CCL21, without affecting the chemotaxis toward CCL19 (Figure 2D). In conclusion, these results indicate that both NRP2a and NRP2b enhance the CCL21-directed migration of mDCs with a comparable efficiency and that the reinforcing effect requires the presence of PSA on the NRP2 polypeptide chain.

PSA controls DC migration in vivo

To investigate the in vivo relevance of the above-described PSA-mediated enhancement of CCL21-driven chemotaxis, we assayed the migration of human mDCs to lymph nodes in a xenograft mouse model. Thereby, we made use of an experimental setup earlier shown by Helfer et al. (2010) and by Briley-Saebo et al. (2010) to preserve the migratory potential of human mDCs in vivo.

mDCs generated from independent donors to be used for the in vivo adoptive transfer experiment were treated with EndoN or left untreated (Figure 3A) and tested for their ability to migrate in vitro toward mouse CCL21 and CCL19. As previously observed for human chemokines, the removal of PSA from mDCs by EndoN treatment reduced their in vitro chemotaxis toward murine CCL21, but not toward mouse CCL19 (Figure 3B). Simultaneously to the in vitro chemotaxis experiments, untreated or EndoN-treated CFSE-labeled human mDCs were also injected into distinct footpads of C57BL/6 mice. By flow cytometry, their presence in popliteal lymph nodes was analyzed 18 h after injection. This time is long enough to allow DCs to reach the draining lymph nodes, as previously reported (Briley-Saebo et al. 2010; Helfer et al. 2010) and is short enough to limit endogenous re-expression of PSA in the EndoN-treated DCs, as shown before (Rey-Gallardo et al. 2010) and for the development of an adaptive immune response against the human DCs graft.

Quantification of the number of CFSE-labeled DCs present in lymph nodes revealed in average six times more grafted cells if mDCs expressed PSA (Figure 3C and D). This finding, which perfectly correlates with the contribution of PSA to mDC migration as denoted in the in vitro chemotaxis experiments discussed above (Figure 2D and 3D), strongly indicates that PSA controls in vivo mDC migration to lymph nodes.

Discussion

In this work, we provide experimental evidences indicating that NRP2 is likely the most abundant polysialylated protein present in mDCs. We have also found out that NRP2 is found in human mDCs as two isoforms, NRP2a and NRP2b, that both NRP2 forms can be polysialylated and that their overexpression in DCs specifically enhances DC migration toward CCL21. Interestingly, the enhancement effect was abolished when mDCs overexpressing NRP2 isoforms were devoid of...
PSA after EndoN treatment. These results demonstrate the requirement of NRP2 polysialylation to mediate the enhancing effect on CCL21-directed chemotaxis.

Two major membrane bound NRP2 isoforms have been described, NRP2a and NRP2b, that result from alternative splicing and differ after amino acid 808 (Rossignol et al. 2000). NRP2b has only been described at the amino acid sequence level and, therefore, no data on NRP2b ligands and on the functional properties of this protein are yet available. Since both NRP2 isoforms share most of their extracellular domains, it is reasonable to expect that NRP2b, like NRP2a, binds VEGF and Sema3F (Zachary et al. 2009). On the other
hand, the two proteins are largely different in their transmembrane and cytoplasmic regions (Rossignol et al. 2000), implying different functions upon ligand binding (Zachary et al. 2009). NRP2a for instance has a C-terminal PDZ domain recognition sequence in its cytoplasmic domain which might dictate its interaction with synectin, a protein that binds to G-protein-coupled receptors and modulates signaling.

Although beyond the scope of the current study, an important question to answer in the future is whether polysialylated NRP2a/NR2b maintain their capability to interact with VEGF and Sema3 ligands.

However, it is likely that the addition of PSA to NRP2a and NRP2b equips the molecules with new ligand-binding properties. Important in this regard is the demonstration that PSA added to its major carrier NCAM facilitates the interaction with basic neurotrophins, thus serving as a reservoir or a regulator of the concentration of neurotrophins (Kanato et al. 2008). Likewise, the similar behavior of NRP2a and NRP2b on mDC chemotaxis toward CCL21, which appears to be independent of their respective transmembrane/cytoplasmic regions, favors the hypothesis that PSA attached to NRP2 functions as a binding and not as a signaling, module for the CCL21 chemokine, increasing its local concentration and/or facilitating its presentation to the CCR7 receptor. The described interaction in vitro between PSA and CCL21 (Bax et al. 2009) supports this view. Furthermore, this behavior is reminiscent of that of growth cone collapse mediated by the NRP2-homologous protein NRP1, and its ligand Sema3A, where the deletion of the cytoplasmic domain of NRP1 does not impair the Sema3A-induced growth cone collapse (Nakamura et al. 1998). An open question but certainly not trivial to answer is if NRP2a and NRP2b as well as their polysialylated forms have individual functions. One strategy to approach this complex question may be to study other mDC effector functions that are nonrelated to mDC migration (e.g. DC–T cell interactions could be differentially affected by the two NRP2 isoforms (Curreli et al. 2007).

Our experiments of adoptive transfer of human mDCs into a mouse xenograft model revealed a severe impairment of migration to draining lymph nodes of PSA-lacking human mDCs. The expression of NRP2 in most mDCs carrying PSA suggests that the impaired in vivo migration of DCs devoid of PSA could be largely attributed to polysialylated NRP2. Nevertheless, whether other putative polysialylated proteins could also contribute to either DC migration or other DC effector functions is an interesting issue that warrants further studies.

The attraction of mDCs to the lymph nodes is mostly influenced by CCL21, a chemokine present along all the lymphatic vasculature, whereas CCL19 is mainly expressed in lymph nodes (Randolph, Angeli et al. 2005).
Here, it is important to emphasize that PSA removal from mDCs seems to cause a greater impairment on in vivo mDC migration to lymph nodes than on in vitro chemotaxis toward CCL21, suggesting that the role of PSA in the control of mDCs migration in vivo might have more relevance than that previously argued on the basis of the in vitro chemotaxis experiments. This observation is of major relevance as the animal model system reflects the summary effect of the complexity of the in vivo system, where besides of chemotaxis, other processes like cell–cell and cell–extracellular matrix interactions are likely to affect cell migration. Important in this context, DC migration to lymph nodes requires also exit from the tissues via the afferent lymphatics and the transmigration across the lymphatic vessel endothelium, a process that is favored by the CCL21-mediated activation of LFA-1 in mDCs (Eich et al. 2010; Johnson and Jackson 2010). It seems allowed to hypothesize that the higher ability of PSA-bearing mDC to interact with CCL21 results in a greater LFA-1 activation, and therefore in an increased lymphatic transmigration of mDCs, finally leading to the elevated numbers of cells observed in the lymph nodes (Figure 3C and D).

The described role of PSA in disrupting cell–cell and cell–extracellular matrix interactions might also contribute to mDC migration in the in vivo setup (Yang et al. 1994; Johnson et al. 2005). In this regard, PSA coupled to NCAM has been suggested to modulate NCAM-mediated cell adhesion during development (Weinhold et al. 2005) and invasion of glioma cells in the brain (Suzuki et al. 2005). In addition, PSA has been reported to control migration of T cell progenitors from the bone marrow to the thymus by increasing their capacity to escape from the bone marrow niches (Drake et al. 2009). Therefore, it is tempting to speculate that PSA, besides regulating mDC chemotaxis toward CCL21, can also modulate other chemotaxis-independent processes involved in mDC migration to lymph nodes.

The use of the in vivo experimental setup that we report in this current study should constitute a suitable tool to test on how drugs targeted to control the expression and function of any of the components of the CCR7/CCL21/NRP2/ST5SiaIV axis might affect human mDC migration. In summary, our data pointing out PSA as a crucial factor for mDC migration offer a new molecular approach to regulate mDC migration and thereby immunity.

Materials and methods

Reagents

Human and mouse CCL21 and CCL19 chemokines were obtained from PreproTech. Antibody against NRP2 was from R&D Systems. This antibody was used at 10 µg/mL for flow cytometry experiments and at 0.1 µg/mL for western blot. For PSA detection, the monoclonal antibody 735 was used (Frosch et al. 1985). Antibody 735 was used at 10 µg/mL for flow cytometry experiments and at 0.5 µg/mL for western blotting analysis. Antibody against tubulin was from Sigma. The following secondary antibodies were used: anti-mouse IgGs/HRP (Dakocytomation) for western blotting analysis using antibody 735; donkey anti-mouse IgG/FITC for flow cytometry analysis using antibody 735; donkey anti-mouse Cy5 (Jackson Laboratories) for double-staining flow cytometry analysis using antibody 735; donkey anti-goat IgG/HRP (Santa Cruz Biotechnology) for western blotting analysis using the NRP2 antibody; chicken anti-goat Alexa 488 (Invitrogen) for cytometry analysis using the NRP2 antibody. All of them were used at the concentrations recommended by the manufacturer. Plasmids pcDNA1-ST8SiaIV and pcDNA3.1-hygro-NRP2a were kindly provided by Drs Minoru Fukuda and Gera Neufeld, respectively. EndoN was purified as described (Hallenbeck et al. 1987) and was generously provided by Dr. Urs Rutishauser.

Generation of monocyte-derived DCs

Monocyte-derived DCs were generated from peripheral blood mononuclear cells of healthy donors as described (Puig-Kroger et al. 2006). For maturation, immature DCs were treated with 100 ng/mL of LPS (Escherichia coli 0111: B4, InvivoGen) for the times indicated.

Flow cytometry analysis of mDCs double stained with PSA and NRP2 antibodies

Immature DCs were matured for 48 h with LPS at 100 ng/mL. Cells were collected and double stained for NRP2 and PSA (735 antibody) using as secondary antibodies chicken anti-goat Alexa 488 (Invitrogen) and donkey anti-mouse Cy5 (Jackson Laboratories), respectively. Appropriate isotype control antibodies were used to select plot gates. Stained cells were analyzed with a Beckman-Coulter FC500 cell analyzer.

RT-PCR for NRP2 isoform detection

cDNAs were synthesized using random hexanucleotides from RNA (isolated with RNeasy columns (Qiagen)) obtained from mDCs generated from either independent donors or the A375 and tumor cells lines using the High Fidelity cDNA synthesis Kit (Roche). NR2a and NR2b were simultaneously amplified. The sense oligonucleotide 5′-GGAGTGA TagGGAAA GGACGTT-3′ was used for the amplification of both NR2a and NR2b. The antisense oligonucleotides used were 5′-AA AAAGAGTGGCTGTACACCT-3′ for NR2a and 5′-CAAG AAGACCGATCCTCCT-3′ for NR2b. Amplification reactions were performed in the presence of 1 M betaine in the PCR cocktail to facilitate the denaturation of GC-rich regions and were performed according to the following thermal cycle: 30 s at 94°C, 30 s at 63°C and 30 s at 72°C for 35 cycles. The amplified fragments were resolved by agarose gel electrophoresis. Expected sizes of the PCR products were of 290 bp for NR2b and 250 bp for NR2a. Control PCRs were performed using oligonucleotides 5′-GGCTGAGGAA CGGGAAGCTTGTCA-3 and 5′-CggCCA TCACGCCACAG GACGTT-3 for NRP2b. Amplification reactions were performed in the presence of 1 M betaine in the PCR cocktail to facilitate the denaturation of GC-rich regions and were performed according to the following thermal cycle: 30 s at 94°C, 30 s at 63°C and 20 s at 72°C for 35 cycles. The amplified fragments were resolved by agarose gel electrophoresis. Expected sizes of the PCR products were of 290 bp for NR2b and 250 bp for NR2a. Control PCRs were performed using oligonucleotides 5′-GGCTGAGGAA CGGGAAGCTTGTCA-3 and 5′-CggCCA TCACGCCACAG GACGTT-3, which together amplify a 417-bp fragment from the glyceraldehyde-3-phosphate dehydrogenase mRNA.

Construction of the expression vector encoding the NRP2 isoform

The entire open reading frame of human NRP2b was amplified by PCR from cDNA generated from human DCs mature with LPS for 48 h, using the following primers: sense: 5′-CGGAAGCTTACACTCTCCTCAAAATGGAATATGTTTCC'T
CTCACC-3′ and antisense: 5′-GGCTCTAGACCTCAGCAGTGCGAGCCACGGTC-3′. The amplification reaction was performed in the presence of 1 M betaine in the PCR cocktail to facilitate the denaturation of GC-rich regions. Amplification was performed according to the following thermal cycle: 30 s at 95°C, 30 s at 62°C and 60 s at 72°C for 10 cycles, followed by 30 s at 95°C, 30 s at 62°C and 60 s at 72°C (with 1 s of additional extension per cycle) for 25 cycles. The PCR product was subcloned into the HindII/XbaI sites of pcDNA3.1-hygro vector (Invitrogen). The identity of NRP2b was confirmed by DNA sequencing.

**Transfection of COS7 cells**

COS7 cells cultured in 10 cm plates at 80% of confluence were co-transfected with 18 µg of pcDNA1-ST8SiaIV and either with 6 µg of pcDNA3.1-hygro (used as control), with 6 µg of pcDNA3.1-hygro-NRP2a or with 6 µg of pcDNA3.1-hygro-NRP2b, using Lipofectamine 2000 as recommended by the manufacturer. Twenty-four hours later, cells were analyzed by flow cytometry and western blot.

**Transfection of K562 cells**

2 × 10^6 K562 cells were co-transfected with 2 µg of pcDNA1-ST8SiaIV and either with 2 µg of pcDNA3.1-hygro (used as control), with 2 µg of pcDNA3.1-hygro-NRP2a or with 2 µg of pcDNA3.1-hygro-NRP2b, using the Solution V nucleofector kit (Lonza), following the manufacturer’s instructions.

**EndoN treatment of DCs and transfected COS7 and K562 cells**

mDCs, COS7 or K562 cells at 2 × 10^6 cells/mL were incubated for 1 h at 37°C with either 20 U of EndoN at 1 U/µL or an equal volume of PBS/glycerol in RPMI 1640, with occasional shaking. Afterward, cells were washed with complete medium. PSA removal after EndoN treatment was tested by western blotting, and when indicated by flow cytometry using the monoclonal antibody 735.

**Nucleofection of NRP2a and NRP2b expression plasmids in DCs**

4 × 10^6 immature DCs were transfected with 5 µg of plasmid (pcDNA3.1-hygro or pcDNA3.1-hygro-NRP2a or pcDNA3.1-hygro-NRP2b) using the Lonza human dendritic cell nucleofector kit, following the manufacturer’s instructions. One hour later, DCs were matured by the addition of 100 ng/mL of LPS. In vitro chemotaxis assays were carried out 18 h later. Protein expression levels for NRP2 were assayed by flow cytometry and western blotting using the anti-NRP2 antibody. PSA expression levels were monitored by using antibody 735.

**CSFE labeling of DCs**

Untreated or EndoN-treated DCs at 1 × 10^6 cells/mL were incubated in PBS + 0.1% BSA with 1 µM CSFE (Invitrogen) for 30 min at 37°C. Labeling was stopped by the addition of five volumes of complete culture medium at 4°C and incubation for 5 min on ice. After two washes, cells were resuspended in PBS for flow cytometry analysis or in RPMI 1640 for the in vivo migration assays.

**In vivo migration assay**

2 × 10^6 CSFE-labeled mDCs treated or untreated with EndoN were resuspended in 25 µL of RPMI 1640 and injected subcutaneously in distinct footpads of C57BL/6 mice. Eighteen hours later, animals were killed and the draining popliteal lymph nodes were extracted. Single-cell suspensions were prepared by mechanical disaggregation and analyzed by flow cytometry to identify CFSE-labeled cells.

**Western blotting**

For western blotting analysis, 10 µg each cell lysates were subjected to SDS–PAGE under reducing conditions and transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore). Western blots were developed with appropriate HRP–conjugated secondary antibodies and an ECL substrate (Pierce) detection system.

**In vitro chemotaxis assays**

Chemotaxis experiments of DCs toward human and mouse chemokines CCL21 and CCL19 were performed as described (Rey-Gallardo et al. 2010). All chemokines were used at 200 ng/mL.

**Statistical analysis**

Statistical analyzes were performed using the paired Student’s t-test.

**Supplementary data**

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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**Conflict of interest**

None declared.

**Abbreviations**

DC, dendritic cell; PSA, polyasialic acid; NRP2, neuropilin-2; LPS, Lipopolysaccharide; RT-PCR, reverse transcriptase-polymerase chain reaction; PBS, phosphate buffer saline; CFSE, carboxyfluorescein succinimidyl ester; SDS-PAGE,
sodium docecyl sulfate polyacrylamide gel electrophoresis; HRP, horseradish peroxidase.

References


