Human dendritic cells express neuronal Eph receptor tyrosine kinases:
role of EphA2 in regulating adhesion to fibronectin

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ABSTRACT

Eph receptor tyrosine kinases and their ligands, the ephrins, have been primarily described in the nervous system for their role in axon guidance, development and cell intermingling. Here we addressed whether Eph receptors may also regulate dendritic cells (DC) trafficking. RT-PCR analysis showed that DC derived from CD34\(^+\) progenitors, but not from monocytes, expressed several receptors, in particular EphA2, -A4, -A7, -B1, and EphB3 mRNA. EphB3 was specifically expressed by Langerhans cell type, and EphA2 and EphA7 by both Langerhans- and interstitial-type DC. EphA and EphB protein expression on \textit{in vitro} generated DC was confirmed by staining with ephrin-A3-Fc and ephrin-B3-Fc fusion proteins that bind to different Eph members, in particular EphA2 and EphB3. Immunostaining with anti-EphA2 antibodies demonstrated the expression of EphA2 by immature DC and by \textit{ex vivo} isolated skin Langerhans cells. Interestingly, ephrin expression was detected in epidermal keratinocytes and also in DC. Adhesion of CD34\(^+\)-derived DC to fibronectin, but not to poly-L-lysine, was increased in the presence of ephrin-A3-Fc, a ligand of EphA2, through a \(\beta1\) integrin activation pathway. As such, EphA2/ephrin-A3 interactions may play a role in the localization and network of Langerhans cells in the epithelium or/and in the regulation of their trafficking.

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INTRODUCTION

Dendritic cells (DC) are antigen presenting cells (APC) that are present in all lymphoid and non lymphoid organs, and have the unique capacity to activate naive T cells \(^1,2\). DC originate from bone-marrow and migrate as precursors through the bloodstream to non-lymphoid tissues. Tissue DC (e.g. the epidermal Langerhans cells) are at an immature stage and able to capture antigens with high efficiency. Antigen-bearing cells migrate from the periphery towards lymphatic vessels to reach the lymphoid tissues and localize in T cell-rich areas as mature interdigitating DC (IDC) \(^3-5\). At this site, IDC efficiently present the processed antigens to naive T cells and induce specific immune responses \(^1\). Thus, migration constitutes an integral part of DC function. The recruitment of DC into tissue damage site and the subsequent migration of DC into secondary lymphoid organs relies on a dynamic and complex series of events involving cell surface receptors, primarily selectins, and also integrins, which mediate the initial engagement of rolling cells \(^6,7\). Cell activation by signaling molecules, such as chemokines, leads to activation of adhesion molecules, and the high affinity binding of activated integrins to endothelial ligands, or extracellular matrix proteins, produces tight adhesion, shape change, and diapedesis to localize in extravascular foci. Although the role of chemokines in DC migration have been studied in depth \(^8\), the role of other molecules in regulating their tissue trafficking remains to be investigated.

Interactions of Eph receptor tyrosine kinases with their membrane-bound ligands, the ephrins, are implicated in important developmental processes including tissue morphogenesis, control of angiogenesis and axonal guidance \(^9,10\). According to their structural features and their preference for different ephrins, Eph receptors have been divided in two groups: EphA (A1 to A8) receptors bind preferentially glycosylphosphatidylinositol (GPI)-anchored
ligands, the ephrin-A (A1 to A5), whereas EphB (B1 to B6) bind transmembrane ligands, the ephrin-B (B1 to B3) \(^{11,12}\) that are phosphorylated after Eph-ephrin interaction \(^\text{13}\). An individual Eph receptor, however, has a wide variation in affinity for different ephrins \(^{11}\): e.g. ephrin-A3 binds to EphA3, EphA2, EphA7, EphA5 and EphA4 with decreasing affinities \(^{11,12}\). Ephrins play important roles during axon guidance by providing a repulsive guidance signal to Eph receptor cells: navigating growth cones or migrating cells expressing Eph receptors turn away from cells expressing the cognate ephrin ligand \(^\text{14}\). Alternatively, ephrins can stimulate the assembly of endothelial cells to form blood vessels, strengthening the dual role of ephrins \(^{15-17}\). A characteristic of the ephrin-Eph signaling system is the ability to elicit bidirectional signaling that leads to restriction of cell migration and cell intermingling as observed during hindbrain segmentation and of vasculogenesis, possibly via modulation of integrin function \(^{15,18,19}\). Several studies, albeit conflicting, have described a role for Eph molecules in the regulation of cell adhesion and the cytoskeleton \(^{20-22}\).

To date, a few Eph receptor members have been sporadically found in cells of the human hematopoietic system: EphB1 is abundantly detected in plasmacytoid DC \(^\text{23}\), EphB4 has a wide tissue distribution and includes several myeloid hematopoietic and human CD34\(^+\) cells \(^{24}\); EphB6 is expressed by CD4\(^+\)CD8\(^-\) thymocytes and by peripheral T lymphocytes \(^{25}\); EphA4 and EphA7 have been also described during B cell differentiation \(^{26}\); EphA2 mRNA is found in skin, and lymphoid tissues \(^{27,28}\). Nevertheless, despite the expression of some Eph receptors in hematopoietic cells and the recent description of EphB6 role in T cell costimulation \(^\text{29}\), their functions are largely unknown in the immune system.

In the present study, we report a comparative analysis of the expression of Eph members in cells from the hematopoietic system and we focus our attention on their expression in human DC. We found that DC derived \(\textit{in vitro}\) from cord blood CD34\(^+\) progenitor cells cultured with GM-CSF and TNF\(\alpha\) for 12 days...
express EphA2, -A4, -A7, -B1, and EphB3 mRNA but not EphA1, -A3, -A5, -B2, -B4 and -B6. Interestingly, EphA2 and EphA7 are preferentially expressed by DC, and EphB3 is restricted to Langerhans cell type. Using ephrin-A3-Fc and ephrin-B3-Fc fusion proteins, we confirmed the strong expression of EphA and EphB protein at the cell surface of CD34⁺-derived DC, and demonstrated expression of EphA2 protein in immature DC. Finally, we report functional evidence for the role of Eph-ephrin interactions in the regulation of integrin-mediated DC adhesion to fibronectin.
METHODS

Hematopoietic factors, reagents, cells and cell lines.
rhGM-CSF (specific activity: $2 \times 10^6$ U/mg; Schering-Plough Research Institute, Kenilworth, NJ), rhTNFα (specific activity: $2 \times 10^7$ U/mg; Genzyme, Boston, MA), rhSCF (specific activity: $4 \times 10^5$ U/mg; R&D Systems, Abington, U.K.) and rhIL-4 (specific activity: $10^7$ U/mg; Schering-Plough Research Institute) were used at 100, 2.5, 25 and 10 ng/ml respectively.

Peripheral Blood Mononuclear Cells (PBMC), blood T cells, monocytes, and tonsil B cells were purified as previously described in details. Granulocytes were generated in vitro from CD34+ progenitors in the presence of G-CSF and SCF for 12 days. T cells were activated with plastic-coated anti-CD3 and soluble anti-CD28 mAb for 3, 12 and 24 h. Other cells were unactivated or activated by PMA-ionomycin for 1 h and 6 h (PMA: 1 ng/ml, Sigma, St Louis, MO; Ionomycin: 1 µg/ml, Calbiochem, La Jolla, CA) and pooled. Murine fibroblasts transfected with human CD40 ligand (CD40L L cells) were produced in the laboratory. Langerhans cell-enriched epidermal cell suspensions were prepared from normal human skin as described. All cell types were cultured in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Flow laboratories, Irvine, UK), 10 mM Heps, 2 mM L-glutamine, 100 µg/ml gentamicin (Schering-Plough, Levallois-Perret, France) (hereafter referred to as complete medium).

Immunohistochemistry and adhesion assays were performed using chimera of human ephrin-A3 and ephrin-B3 fused to human IgG Fc (R&D Systems, Minneapolis, MN), or chimera of human CD152 (CTLA-4) or CD95 (Fas) fused to murine and human IgG Fc respectively (Ansell Corp., Bayport, MN and R&D Systems). Ephrin-A3-Fc can interact with multiple EphA receptors, in particular EphA3 and EphA2 with a high affinity, and EphA7, EphA5 and EphA4 with...
decreasing affinity, and ephrin-B3 can interact with EphB1, EphB2 and EphB3, and also EphA4 with a wide variation of affinity \(^{11,12}\).

**Generation of DC from CD34\(^+\) progenitors and monocytes.**

Umbilical cord blood samples were obtained according to institutional guidelines. CD34\(^+\) progenitors were isolated using Minimacs separation columns (Miltenyi Biotec, Bergish Gladbach, Germany) as described \(^{32}\). In all experiments the isolated cells were 80 to 99% CD34\(^+\) as judged by staining with anti-CD34 mAb. Cultures of CD34\(^+\) cells were established in the presence of SCF, GM-CSF, TNF\(\alpha\) and 2.5% AB\(^+\) human serum as described \(^{32,33}\). Cells collected after 6 days of culture were further cultured in the presence of GM-CSF, and TNF\(\alpha\) until day 11-12 when 70-90% of cells are CD1a\(^+\) DC. In some experiments, cells were separated at day 6 according to CD1a and CD14 expression into CD14\(^+\)CD14\(^-\) and CD14\(^-\)CD14\(^+\) using a FACStar\(^+\)® cell sorter (Becton Dickinson, Mountain View, CA) and cultured until day 12 with GM-CSF and TNF\(\alpha\) as described \(^{32}\). In some instances, cells were activated with LPS at 20 ng/ml (Sigma) in the presence of GM-CSF until day 14.

Monocytes were purified by immunomagnetic depletion (Dynal, Oslo, Norway) of low density PBMC isolated on a 52% Percoll gradient. The depletion was performed with anti-CD3, anti-CD19 and anti-CD8 mAbs produced in the laboratory, and with purified anti-CD56 and anti-CD16 mAbs (Beckman Coulter, Miami, FL). Monocyte-derived DC were produced by culturing purified monocytes for 6 days in the presence of GM-CSF and IL-4 \(^{34}\). Cells were activated with LPS at the concentration of 20 ng/ml for 1h to 72h or with CD40L-transfected L cells (one L cell for five DC) \(^{30}\).

**RT-PCR analysis.**

Total RNA extracted from 1 to 10.\(10^6\) cells \(^{30}\) were treated with DNase I for 30 min at 37\(^\circ\)C (Promega Biosciences, San Luis Obispo, CA). The absence of
genomic DNA was controlled by PCR on RNA preparations using the following β actin primers: sense (exon 2) ATCTGGCACCACACCTTCTA; antisense (exon 3) AATGTCACGCACGATTTCCC. RNA samples were further reverse transcribed using random hexamer primers (Pharmacia, Upsalla, Sweden) and the Superscript RNase-H reverse transcriptase (GIBCO BRL). PCR were performed in a 100 µl volume using 50 ng cDNA, 10 µl 10X PCR reaction buffer (Perkin Elmer Cetus, Norwalk, CT), 2.5 U of Taq polymerase (Gene Amp PCR reagents kit : Perkin Elmer Cetus) and 200 mM dNTPs and 500 nM of the 5’ and 3’ amplification primers. The PCR reactions were made in a DNA thermal cycler (Perkin Elmer) for 35 cycles (1 min denaturation at 94°C, 1 min annealing at 60°C, and 2 min elongation at 72°C). β-actin RT-PCR was used as positive control for the efficiency of the reaction using sense and antisense primers (Stratagene, La Jolla, CA). All cDNA samples were normalized according to the results of β-actin PCR amplification of 21, 28 and 35 cycles (data not shown).

RT-PCR of the different Eph mRNA was performed with the following primers:

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eph A2</td>
<td>241-259 CTCACACACCCGTATGGCAA</td>
<td>976-957 GGCTCTCAGATGCCTCAAAC</td>
<td>736</td>
</tr>
<tr>
<td>Eph A4</td>
<td>54-73 CGCCCTATTTCGTGTCTCT</td>
<td>666-647 CAGATTGCGGACTGTGAGTG</td>
<td>613</td>
</tr>
<tr>
<td>Eph A7</td>
<td>1027-1046 GGGTTCTACAAGTCTTCTC</td>
<td>1538-1519 TCCTTCATTACTCCGCTC</td>
<td>512</td>
</tr>
<tr>
<td>Eph B1</td>
<td>577-597 GTCATTCGCCACCAAGAAGTC</td>
<td>1015-996CCA CGCTTGTCTCAGGCTCA</td>
<td>439</td>
</tr>
<tr>
<td>Eph B2</td>
<td>471-491 CACCAAGACCTTCCCCAACT</td>
<td>905-887 GACGGTGCCATTCTCAACGG</td>
<td>436</td>
</tr>
<tr>
<td>Eph B3</td>
<td>670-690 ACCACGCA GGCTTCGCACT</td>
<td>1408-1389 GGCCTAGTGTGGGCACCTCA</td>
<td>739</td>
</tr>
<tr>
<td>Eph B6</td>
<td>824-843 TCCTGGTGTCCTCAGTTCTG</td>
<td>1475-1456GAAGCAAGGATCGGACAC</td>
<td>652</td>
</tr>
</tbody>
</table>

All Eph primers (except EphB1 and EphB2) allow to distinguish cDNA and genomic DNA.
Flow cytometry analysis.
For cell surface labeling, cells were stained with biotinylated CD152-Fc (CTLA-4-Fc) or CD95-Fc (Fas-Fc) (Ancell Corp.), or biotinylated human ephrin-A3-Fc and ephrin-B3-Fc (R&D Systems), or biotinylated human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) (5 µg/ml). Then, the cells were incubated with PE-conjugated streptavidin (DAKO, Copenhagen, Denmark). For EphA2 cell surface labelling, cells were stained with a monoclonal antibody specific for human EphA2 (20 µg/ml) (clone D7, Sigma) and revealed with PE-conjugated goat F(ab’)2 anti-mouse Ig (DAKO) according to standard techniques. Negative control was performed with an unrelated isotype-matched murine mAb (DAKO).

For intracellular staining of rabbit polyclonal antibodies against intracellular epitopes, cells were first permeabilized for 15 min with permeabilization medium (0.1% saponin, 1%FCS), and then incubated with the following antibodies (20 µg/ml) : anti-EphA2 (C-20), anti-ephrin-A3 (K19), anti-ephrin-B1 (C18), anti-ephrin-B2 (P20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-CD4 (Immunodiagnostics, Inc., Woburn, MA) or unrelated rabbit IgG (Jackson ImmunoResearch Laboratories) for 30 min at 4°C in the presence of permeabilization medium. After two washes, cells were incubated with PE-anti-rabbit IgG (Sigma). For double color immunofluorescence staining, after the first step, the cells were incubated for 30 min in 2% normal mouse serum (DAKO) in PBS, and then surface labeled with FITC-CD1a (BD Biosciences Pharmingen, Erembodegen, Belgium), FITC-HLA-DR or FITC-CD86 (Becton Dickinson) according to standard techniques. Negative controls were performed with FITC-conjugated unrelated isotype-matched murine mAbs (DAKO). Fluorescence was analyzed with a FACScan® flow-cytometer (Becton Dickinson).
Immunocytochemistry and immunohistochemistry.

Cytospins of CD34⁺-derived DC or frozen 6 µm tissue sections of human normal skin were fixed and permeabilized with acetone at –20°C for 30 min. To block endogenous non specific activities, cytospins or sections were pre-treated with Power block™ universal blocking reagent, followed by avidin and biotin block solutions (InnoGenex, San Ramon, CA) for 15 min each step at room temperature (RT). After being washed in PBS, the coverslips were incubated for 30 min at RT in 2% normal goat serum (DAKO) (same species as secondary antibody). Then they were stained for 1 h at RT in a humid atmosphere with the following rabbit polyclonal antibodies (20 µg/ml) prepared in 2% normal goat serum in PBS: anti-EphA2 (C-20), anti-ephrin-A3 (K19), anti-ephrin-B1 (C18), anti-ephrin-B2 (P20) (Santa Cruz Biotechnology), anti-CD4 (Immunodiagnostics) or unrelated rabbit IgG (Jackson ImmunoResearch Laboratories). After a wash in PBS, the coverslips were incubated for 30 min at RT with biotinylated goat anti-rabbit IgG (H+L) (Vector Laboratories, Inc., Burlingame, CA) and then with streptavidin alkaline phosphatase (Biosource International, Camarillo, CA). Alkaline phosphatase activity was revealed using alkaline phosphatase substrate III (Vector Laboratories) for 1-10 min at RT. For double staining, sections were simultaneously stained with anti-EphA2 polyclonal antibody and DCGM4 mAb (produced in the laboratory), and revealed sequentially with biotinylated goat anti-rabbit IgG (H+L) (Vector Laboratories) and with peroxydase labeled anti-mouse IgG1 (Binding sites, Birmingham, UK). The biotinylated goat anti-rabbit IgG was revealed with streptavidin alkaline phosphatase following by specific substrate as described above. The peroxydase was revealed using 3-amino-9-ethylcarbazole (AEC) substrate (Vector Laboratories) for 5-10 min at RT.
Adhesion assays.

The assays were performed according to previously described protocols \(^{20,35}\) with modifications. 96-well flat bottom plates (NUNC, Roskilde, Denmark) were coated with 5 µg/ml or serially diluted (from 20 to 1 µg/ml) fibronectin or poly-L-lysine (Sigma) O/N at 4°C. Non-specific binding sites were blocked at RT for 1h using 1%BSA/PBS. Human ephrin-A3-Fc, ephrin-B3-Fc, ephrin-A4-Fc, Fas-Fc (R&D Systems) or human IgG\(_1\) (Jackson ImmunoResearch Laboratories) were deposited at a concentration of 5 µg/ml, or from 1 to 20 µg/ml into the plates. Soluble Arginyl-glycyl-aspartic acid peptide (RGD) (Calbiochem GmbH, Schwalbach, Germany) was added in plates at 0.01 to 10 µM. The cultured cells were washed once, and resuspended in serum free RPMI medium. 1x10^5 cells in 100 µl were plated per well (in triplicates) and centrifuged at 1200 rpm for 3 min. The medium was discarded and remaining cells were washed gently once with PBS (without Ca\(^{2+}\) and Mg\(^{2+}\)) at RT. Subsequently, the cells were fixed with 0.5% paraformaldehyde/0.5% glutaraldehyde (Sigma) at RT for 30 min, and stained with 0.5% crystal violet (Merck Eurolab GmbH, Darmstadt, Germany) in 20% methanol at RT for 10 min. The cells were washed 3 times with water and extracted with 50% ethanol/50 mM sodium citrate pH=4.5 (50µl/well). OD was measured at 570 nm.
RESULTS

CD34⁺-derived DC express high levels of mRNA for protein tyrosine kinase receptors of the Eph family.

In order to determine whether Eph receptors were expressed by human DC, we analyzed by RT-PCR the expression of EphA and EphB members in different cDNA samples from DC and other hematopoietic cells that were previously normalized according to the results of β-actin PCR amplification and checked for the absence of genomic contamination (Figure 1, lower panels). We compared this expression in two types of in vitro generated DC, DC obtained from monocytes cultured in the presence of GM-CSF and IL-4 for 6 days, and DC obtained from CD34⁺ progenitors cultured with GM-CSF and TNFα for 6 and 12 days. DC were also further activated by hCD40L expressing L cells. Interestingly, cDNA encoding some Eph members in particular EphA2, EphA4, EphA7, EphB1, EphB3 and more weakly EphB2 and EphB6 were amplified in day 12 CD34⁺-derived DC; in contrast, in monocyte-derived DC only EphA4, after activation of the cells by CD40L, and to a weaker extent, EphB6, were detected (Figure 1). Messengers for EphA1, -A3, -A5, and -B4 were not detected in in vitro generated DC after 35 cycles RT-PCR (data not shown). EphA2 expression seems to be restricted to DC, as only a significant level of messenger was detected in CD34⁺-derived DC and a very faint band was present in freshly isolated monocytes, in CD40L-activated monocyte-derived DC and in PBMC but not in the other hematopoietic cells tested. Similarly, EphA7 was only detected by RT-PCR in day 12 CD34⁺-derived DC. EphB3 was strongly expressed in DC, and weakly in B cells and in PBMC. EphA4 and
EphB1 mRNA, both present in DC, were also detected in other hematopoietic cells. Furthermore, the expression of Eph members, in particular, EphA2, EphA7, EphB1, and EphB6 was most abundant in relatively immature DC recovered after 12 days of culture, but decreased in fully activated DC recovered after 4 further days of co-culture with hCD40L-transfected fibroblasts. 

Taken together, these results reveal for the first time that DC express receptor protein tyrosine kinases of the EphA and EphB families, originally described in the nervous system for their role in axon guidance.

**EphA2 and EphA7 mRNA are present in different types of DC but EphB3 transcript is specifically present in Langerhans cells.**

Given the expression of Eph messenger in *in vitro* CD34⁺-derived DC, we next wondered whether Eph expression might correlate with a particular type of DC. Thus, we analyzed by RT-PCR the expression of the most DC restricted Eph members in either CD1a⁺- or CD14⁺-derived DC in GM-CSF and TNFα culture conditions. Only the CD1a⁺ subset has been shown to differentiate into Langerhans cells, characterized by the presence of Birbeck granules and the expression of E cadherin, in the absence of exogenous TGFβ³⁶; the CD14⁺ subset being more closely related to interstitial DC³². CD1a⁺ and CD14⁺ precursors were FACS® sorted at day 6 and recultured with GM-CSF and TNFα for 6 additional days. The presence of Eph transcripts in the two purified subsets was compared to total CD34⁺-derived DC (corresponding to unsorted cells), using samples previously normalized according to the results of PCR amplification of β-actin (Figure 2) and checked for the absence of genomic contamination (data not shown). Analysis of EphA2 and EphB1 transcripts by RT-PCR showed a strong signal in both CD1a⁺ and CD14⁺-purified DC (Figure 2). In contrast, EphA7 mRNA was most abundant in CD1a⁺-derived DC.
compared to the CD14$^+$ subset. Interestingly, EphB3 was only detected in CD1a$^+$-derived DC but not in CD14$^+$-derived DC. To confirm the restricted expression of EphB3, we performed RT-PCR in ex vivo isolated skin Langerhans cells. As shown in Figure 2, whereas basal keratinocytes did not express EphB3 mRNA, the transcript was amplified in fresh Langerhans cells isolated from epidermis. Therefore, although two other Eph members (EphA2, EphA7) are also strongly expressed by CD34$^+$-derived DC, EphB3 represents the most Langerhans cell restricted Eph member.

**Expression of EphA and EphB protein is regulated during DC differentiation.**

In order to study the overall expression of Eph proteins during the culture of human CD34$^+$ cord blood progenitors with GM-CSF and TNF$\alpha$, we next used a biotinylated ephrin-A3-Fc fusion protein (composed of the extracellular domain of ephrin-A3 fused to the Fc fragment of a human IgG1) and a biotinylated ephrin-B3-Fc fusion protein. Indeed, due to the considerable promiscuity in interactions among ephrin ligands and Eph receptors, ephrin-A3 can interact with multiple EphA receptors, in particular EphA3 and EphA2 with a high affinity, and EphA7, EphA5 and EphA4 with decreasing affinity, and ephrin-B3 can interact with EphB1, EphB2, and EphB3 and also EphA4, with a wide variation in affinity $^{11}$. In agreement with the RT-PCR data (Figure 1), flow cytometry analysis showed a very weak expression of EphA and EphB proteins (17-18%) at the immature stage of DC differentiation (Figure 3, day 6). Expression of both EphA and EphB further increased during DC differentiation until day 11-12. Indeed, the expression of EphA was optimal at day 11-12 of the DC culture (88-79%) and the expression of EphB increased from day 10 to day 12 (56 to 77%) (Figure 3). The expression of both EphA and EphB was downregulated by further activation of the cells by LPS (only 8 to 13% of expression
maturation of the cells was controlled by the increased binding of CTLA4-Fc to CD80/86 during DC differentiation (Day 10, MFI=274; Day 12, MFI=605; Day 13, Unactivated MFI=1297, Activated MFI=1608, values for the representative experiment in Figure 3). Based on the RT-PCR results, the binding of ephrin-A3-Fc is likely to be primarily mediated through the interaction with EphA2. Furthermore, in agreement with the RT-PCR results, we did not find a significant expression of both EphA and EphB in monocyte-derived DC obtained from culture of monocytes with GM-CSF and IL-4 for 6 days (data not shown). The specificity of Eph expression in cultured CD34+-derived DC was further studied by FACS® analysis or immunocytochemistry using a rabbit polyclonal antibody, specific for an EphA2 intracellular epitope. EphA2, absent at immature stage of DC differentiation (day 6), was induced during DC differentiation (day 10-12) (Figure 4A). The expression of EphA2 at the DC cell surface was furthermore shown by staining non-permeabilized DC with a monoclonal antibody specific for EphA2 (Figure 4B). Double staining showed that the EphA2+ cells expressed CD1a and MHC class II, confirming that a significant proportion of in vitro generated DC (46%) expressed EphA2 at day 12 of the culture (Figure 4C). Moreover, EphA2+ cells expressed a low level of CD86 and HLA-DR reflecting their relative immature stage (Figure 4C). In addition, immunocytochemistry showed that day 12 CD34+-derived DC were stained with the anti-EphA2 polyclonal antibody (Figure 4D). The specificity of anti-EphA2 polyclonal antibody was demonstrated by inhibition of staining by the peptide used for immunization. Due to the lack of specific reagents, we have not been able to confirm expression of EphA7 and EphB3 by FACS® analysis or immunocytochemistry.

Consistently with the RT-PCR results, we demonstrated the presence of EphA and EphB at the surface of in vitro generated DC, and identified EphA2 as one of the molecules recognized by the ephrin-A3-Fc fusion protein in DC.
Langerhans cells express EphA2.

The EphA2 expression in in vitro generated CD34⁺-derived DC prompted us to examine the expression of EphA2 in tissues. As it has been previously reported that EphA2 was predominantly expressed in epithelial tissues, in particular by keratinocytes ²⁷, we performed immunohistochemistry on normal skin sections using the polyclonal antibody specific for EphA2 and a monoclonal antibody specific for Langerin, a Langerhans cell specific marker ³⁷. We observed that EphA2 was present in the suprabasal layer of the epidermis, corresponding certainly to keratinocytes (Figure 5A). However, the double staining with Langerin did not allow to formally demonstrate EphA2 expression by Langerhans cells. Next, we confirmed the expression of EphA2 by freshly purified keratinocytes from normal skin by FACS® analysis (Figure 5B). Moreover, we detected the expression of EphA2 by a preparation of freshly purified Langerhans cells from skin whose purity was around 80% as shown by the expression of Langerin (Figure 5C). In another preparation of skin cells, a double staining with EphA2 and CD1a clearly demonstrated that all Langerhans cells (CD1a⁺) and keratinocytes (CD1a⁻) expressed EphA2, confirming the results of immuno-histochemistry (Figure 5D).

Thus, these results establish the expression of EphA2 protein by both Langerhans cells and keratinocytes in normal skin.

Expression of several Eph ligands, the ephrins, in skin.

It has been previously reported that a receptor tyrosine kinase and its cognate ligand can be co-expressed in the same cell ³⁸. Since we observed the expression of EphA2 in CD34⁺-derived DC, we extended the analysis of ephrin in these cells, using polyclonal antibodies specific for ephrin-A3, ephrin-B1 and ephrin-B2 (Figure 6A). We found that ephrin-A3, like its cognate ligand EphA2, was significantly expressed by CD34⁺-derived DC at day 11 (79% of
expression). Ephrin-B1 was detected at a lower level (50% of expression) compared to the positive CD4 control and to the ephrin-A3 staining; ephrin-B2 was not detected in these cells. The specificity of the FACS® staining was confirmed by inhibition of detection in the presence of the immunization peptide. Furthermore, the ephrin-A3 polyclonal antibody, and at a weaker extent, the ephrin-B1 polyclonal antibody stained also cytospin preparations of day 11 CD34⁺-derived DC cultured with GM-CSF and TNFα (Figure 6B). Next, we performed immuno-histochemistry with the different polyclonal antibodies, on serial normal skin sections fixed with acetone. Ephrin-A3, ephrin-B1 and ephrin-B2 were also detected within epidermal keratinocytes, demonstrating that the expression of EphA2 ligand, ephrin-A3, and other ephrins of the B family, match the expression patterns of EphA2 (Figure 6C).

Taken together, these observations reveal the presence of both receptor and ligands in keratinocytes and DC, suggesting that EphA2/ephrin-A3 interaction may play a role in the function, the localization and/or network of Langerhans cells in the epithelium of skin.

**Engagement of Eph receptor by its ligand increases DC adhesion to fibronectin but not to PLL.**

Eph receptors and their ligands have been implicated in integrin-mediated cell adhesion. To investigate the role of Eph receptors expressed by CD34⁺-derived DC in the regulation of cell attachment, we developed an adhesion assay on various components of the extracellular matrix with ephrin-Fc chimera, and IgG or Fas-Fc as negative controls. First, we performed an adhesion assay with day 11 CD34⁺-derived DC stimulated with 10 μg/ml ephrin-A3-Fc or Ig, on plates pre-coated with increasing concentrations of fibronectin (from 0 to 20 μg/ml). The plates were immediately centrifuged for 3 minutes at 1200 rpm. After removing cells in suspension, attached cells were
carefully fixed and stained by crystal violet, and the number of cells was quantified by O.D. reading. As shown in Figure 7A, no spontaneous adhesion of day 11 CD34+ -derived DC to fibronectin or PBS alone was observed when the cells were treated with Ig. However, we found that ephrin-A3-Fc induced increased adhesion to fibronectin reaching its maximum at a concentration of 5 µg/ml. In the next experiments, we used this latter concentration of extracellular matrix to study the effects of other ephrin-Fc chimeras on DC adhesion. Day 11 CD34+ -derived DC were deposited on fibronectin, poly-L-lysine (PLL) (5 µg/ml) or PBS pre-coated plates, with or without increasing concentrations (from 1 to 20 µg/ml) of ephrin-A3-Fc, ephrin-B3-Fc, ephrin-A4-Fc or as negative controls, PBS or Fas-Fc. As shown in one representative experiment in Figure 7B, treatment of DC with 5 µg/ml ephrin-A3-Fc led to a 2.2 ± 0.9 (mean ± SD, n=13) fold increase in adhesion assays to fibronectin compared to Fas-Fc control, which is statistically significant (p<0.001, paired t-test). Stimulation of the cells with ephrin-A4-Fc, another ligand for EphA2 but with a lower affinity, did not significantly increase CD34+ -derived DC adhesion to fibronectin (1.1 ± 0.1 fold, n=3) whereas ephrin-B3-Fc had generally no effect. In the absence of fibronectin, the stimulation of CD34+ -derived DC did not induce adhesion to plastic (Figure 7C) and no increase of the adhesive properties of the DC on PLL-coated plates was observed (Figure 7D). The restricted effect to fibronectin suggested an activation of the integrin system. In order to assess the role of integrins in DC adhesion to fibronectin upon ephrin-A3-Fc stimulation, we next used RGD peptide, the amino acid sequence within fibronectin that mediates cell binding to integrins 40. As shown in Figure 8, ephrin-A3-Fc inducing day 11 CD34+ -derived DC attachment to fibronectin, but not to PLL, was sensitive to competition by the RGD peptide at the concentration of 10 µM. This RGD peptide inhibited the adhesion induced by 20 µg/ml of ephrin-A3-Fc by 72 ± 25% (mean ± SD, n=3) which is statistically significant (p=0.003, n=3). Competitive inhibition experiments using a function-blocking monoclonal
antibody specific to β1 integrin (clone DE9) also blocked Eph-induced CD34+ -derived DC adhesion to fibronectin (data not shown). All together, these data showed that at least integrin β1 was involved in the increased adhesion to fibronectin induced via Eph engagement. Thus, these results strongly support that Eph-ephrin interactions in CD34+ -derived DC contribute to regulation of cell adhesion through an integrin activation pathway.
DISCUSSION

In this study, we present evidence that human DC express several Eph receptors, originally described in the nervous system for their role in the control of axon guidance. We found that EphA2 and EphA7 are preferentially expressed by in vitro generated DC obtained from CD34+ progenitors cultured with GM-CSF and TNFα, and that EphB3 is restricted to Langerhans cell type. Furthermore, we demonstrate a role of ephrin-A3/EphA interaction in the modulation of integrin-mediated DC adhesion to fibronectin.

Despite the large expression of Eph and ephrins in many tissues during development, few studies have reported to date the presence of Eph receptors in cells of the immune system. Here, we found that unexpectedly DC express mRNA for both EphA and EphB members. Some of them, like EphA4, EphB1 and EphB6 are widely expressed by hematopoietic cells, whereas others, like EphA2, -A7 and -B3, are more specific to DC. The restricted expression of EphA7 and EphB3 in DC is strengthened by the presence of these transcripts in immune tissues, such as lymph node and tonsil, and spleen and tonsil, respectively. The expression of EphA2 in DC is in agreement with the presence of the transcript in spleen, lymph node and bone marrow, and confirms the suspected expression of EphA2 by DC. The fact that different Eph members are expressed by CD34+-derived DC suggests that a wide variety of ephrin ligands can interact with Eph-expressed by DC, probably in a tightly regulated mechanism, depending on the ligand-receptor affinity and the level of Eph receptor expression. The Eph-ephrin interaction may also vary...
according to the DC subtype localized in different organs *in vivo*. For instance, among the heterogeneous populations of DC, EphB1 was abundantly found in human plasmacytoid DC, suggesting a specific function of this molecule for this DC subset involved in viral innate immunity. Of interest, expression of most of the Eph receptors shown here, is optimal in immature DC (CD34⁺-derived DC, day 11-12) a stage that could be associated with antigen capture, and corresponds to resident cells found *in vivo* in the periphery, such as Langerhans cells in the epidermis. Indeed, Eph receptors (EphA2, EphA7, EphB3), are strongly expressed in Langerhans cells that reside in the epidermis for a considerable period as sentinels of the immune system, and are down-regulated after stimulation of the cells by an inflammatory stimuli (eg: LPS). In line with these observations, we could not detect any significant Eph messenger in monocyte-derived DC which are more closely related to circulating DC *in vivo*. As both EphA2 and its cognate ligand are expressed by keratinocytes and Langerhans cells, the EphA2-ephrin-A3 interactions could be implicated in the localization and network organization of Langerhans cells in the epithelium of skin in normal conditions. Interestingly, it has been shown that E-cadherin, involved in adhesion of Langerhans cells to keratinocytes, regulates EphA2 tyrosine phosphorylation and localization upon E-cadherin-mediated adhesion in epithelial cells. While E-cadherin stabilizes cell-cell contacts, the interactions between EphA2 and its ligand, active in its membrane-bound form only, are very likely facilitated, resulting in autophosphorylation of Eph receptor that might underlie the inhibition of cell movement, reinforcing probably in this way the network of Langerhans cells and the architecture of the skin. Indeed, Eph/ephrin interaction provokes a bidirectional signaling between adjacent cells that prevent cell intermingling.
between hindbrain segments\textsuperscript{18,19}. Other evidence for a role in controlling cell movement by complementary distribution of Eph and ephrin ligand have been observed in angiogenesis in which ephrin-B2 is present in arteries and EphB4 in veins only\textsuperscript{15}.

Interestingly, we found that in response to ephrin-A3-Fc, but not without stimuli, day 11 CD34\textsuperscript{+}-derived DC are induced to adhere to fibronectin through an integrin-dependent mechanism. Based on RT-PCR data and immunohistochemistry with anti-EphA2 antibodies, it is likely that the observed effect of ephrin-A3-Fc is mediated through interaction with EphA2. Nevertheless, we cannot exclude involvement of other Eph, such as EphA7 and EphA4 that can bind to ephrin-A3-Fc but with a lower affinity than EphA2\textsuperscript{11,12}.

Different studies have shown that Eph receptors regulate either positively or negatively integrin-dependent cell adhesion\textsuperscript{22,39,51}. In view of the current knowledge, at least two alternative mechanisms can be consider. First, specifically oligomerized forms of ephrin-A3-Fc could engage EphA2 on DC resulting in a positive regulation of integrin affinities on fibronectin. This hypothesis is consistent with reported B-ephrin properties in promoting attachment of endothelial cells expressing endogenous EphB1\textsuperscript{52}, via integrin activation. At the opposite, as several reports demonstrate that engagement of Eph deactivates integrins\textsuperscript{53,54}, ephrin-A3-Fc may interrupt a constitutive Eph-ephrin interaction resulting in integrin activation. Indeed, as ephrins are also expressed on DC, ephrin-A3-Fc may compete with ephrin ligands expressed at the DC cell surface that possibly results in a disengagement of the binding of EphA2 to its cognate ligand ephrin-A3 resulting in integrin activation and adhesion to fibronectin. This last scenario is in agreement with reports showing that Eph engagement deactivates integrins; in particular, EphA2 and EphB2.
activation in PC-3 prostate epithelial cells and transfected 293T cells respectively, was shown to induce inhibition of cell adhesion to fibronectin in an integrin dependent manner\textsuperscript{53,54}. These two signalling scenario are possible as both EphA receptors and ephrin-A counter receptors are involved in bidirectional signaling, and seem to be supported by previous descriptions of the role of Eph receptor in the regulation of cell adhesion\textsuperscript{20,21}.

According to the second scenario, one can propose the following hypothesis: after inflammation due to injury or infection, Langerhans cells down-regulate E-cadherin\textsuperscript{55}, and concomitantly decrease EphA2 expression, accounting for their emigration out of the epithelium. Due to down-regulation of Eph expression and absence of ephrins in the surrounding tissue, the Eph are not engaged any longer, allowing for integrin activation and interaction with fibronectin, a component of the extracellular matrix observed in the connective tissue underlying the epithelium\textsuperscript{56}. This interaction with fibronectin is likely to be part of the sequential event for the emigration of Langerhans cells out of the dermis in route to the draining lymph node.

To clarify the mechanism of action of ephrin-A3-Fc, future studies will be necessary to define the role of EphA2 as positive or negative regulator of integrin functions, and to determine whether these properties differ according to the extracellular matrix and to the stage of DC differentiation. As integrins, composed of two chains, $\alpha$ and $\beta$, together with other cell surface adhesion molecules, are required for interactions of cells with other cell types (e.g: endothelial cells), and with extracellular matrix components\textsuperscript{57}, it will be also of interest to identify the specific integrin regulated upon Eph/ephrin interaction.

Taken together, this study shows that Eph molecules, originally involved in axon guidance, are expressed by human DC and regulate DC adhesion. They
might also have the capacity to directly regulate responses to chemokines\textsuperscript{58}, as recently described for other neural guidance molecules\textsuperscript{59,60}. As such, these molecules might be part of important regulatory mechanisms of DC trafficking and might offer avenues to potently manipulate their recruitment \textit{in vivo}. Finally, the present description of Eph molecules in DC complete the restricted list of neuronal molecules expressed by DC, such as neuropilin-1\textsuperscript{61}, and reinforce the similarities between the neuronal and immune system.
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REFERENCES


FIGURE LEGENDS

Figure 1: CD34⁺-derived DC express high levels of mRNA for Eph receptors.

RT-PCR analysis of Eph expression in hematopoietic cells (left panel). cDNA were prepared from unactivated and PMA-ionomycin activated freshly isolated PBMC, blood T cells and monocytes, tonsil B cells or in vitro generated granulocytes. Monocytes, granulocytes and B cells were resting or activated with PMA-ionomycin for 1h and 6h and pooled. T cells and PBMC were activated with PMA-ionomycin for 6h. RT-PCR analysis of Eph expression in in vitro generated CD34⁺-derived DC and monocyte-derived DC (right panel). cDNA were prepared from cord blood CD34⁺ progenitors cultured in the presence of GM-CSF and TNFα for 6 and 12 days, and for an additional 4 days with CD40L L cells, or from monocytes cultured in the presence of GM-CSF and IL-4 for 6 days (unactivated), and following 24h activation with CD40L L cells, or as control, from CD40L L cells. RT-PCR was carried out under standard conditions using 50 ng cDNA for 35 cycles. All cDNA samples were normalized according to the results of β-actin PCR amplification of 21, 28 and 35 cycles (only the amplification of 28 cycles is shown). The absence of genomic contamination was controlled in all RNA samples (before the reverse transcription step) by PCR amplification of β actin with primers designed to amplify genomic DNA as shown in the lower panel. Results are representative of RT-PCR of three independent samples.

Figure 2: Eph expression in different subsets of in vitro generated CD34⁺-derived DC and in vivo isolated skin Langerhans cells.

Cord blood CD34⁺ progenitors were cultured in the presence of GM-CSF, SCF, TNFα and 2.5 % AB⁺ human serum. At day 6, CD1a⁺ and CD14⁺ precursors
were sorted by flow cytometry and recultured with GM-CSF and TNFα for 6 additional days. The presence of Eph transcripts in the two purified subsets was compared to total CD34⁺-derived DC (DC TOT, corresponding to unsorted cells). cDNA were prepared from basal cells and from Langerhans cells, freshly isolated from normal skin. RT-PCR was carried out under standard conditions using 50 ng cDNA for 40 cycles. All cDNA samples were normalized according to the results of β-actin PCR amplification of 21, 28 and 35 cycles (only the amplification of 28 cycles is shown). Results are representative of RT-PCR of three independent samples.

**Figure 3 : The expression of EphA and EphB protein is regulated during DC differentiation.**

Time-kinetics of EphA and EphB expression during CD34⁺-derived DC culture from day 6 to day 14. Flow cytometric analysis was performed on cord blood CD34⁺ progenitors, cultured in the presence of GM-CSF and TNFα until day 12, and after 24h and 48h of LPS activation. Cell surface EphA and EphB expression was followed by staining with biotinylated ephrin-A3-Fc and ephrin-B3-Fc chimera, respectively. As positive control, DC maturation was followed by CD80/CD86 staining using biotinylated CTLA4-Fc chimera. Staining was revealed by PE-conjugated streptavidin. The percent of CTLA4-Fc, ephrin-A3-Fc and ephrin-B3-Fc positive cells is indicated. Dotted-line overlay histograms show non-specific staining with Fas-Fc chimera. Results are representative of FACS® analysis of three independent samples.

**Figure 4 : Expression of EphA2 by CD34⁺-derived DC.**

A- EphA2 expression was analyzed by flow cytometry with anti-EphA2 polyclonal antibody directed against an intracytoplasmic epitope after DC permeabilization with saponin, as described in « Methods ». Cord blood CD34⁺
progenitors were cultured in the presence of GM-CSF and TNFα for 6 to 12 days. Dotted-line overlay histograms represent staining in the presence of the EphA2 peptide used for immunization and bold dotted-line overlay histograms the negative control with an antibody of unrelated specificity.

B- EphA2 expression was analyzed by flow cytometry with an anti-EphA2 monoclonal antibody on non-permeabilized DC (Day 11 with GM-CSF and TNFα). Bold dotted-line overlay histogram shows the staining of an isotype matched control.

C- Double staining of day 11 CD34+ derived DC cultured with GM-CSF and TNFα with surface FITC-CD1a, FITC-HLA-DR or FITC-CD86 antibodies, and intracellular anti-EphA2 polyclonal antibody revealed with PE-anti-rabbit IgG. Quad limits were set up on the isotype matched control dot plot. The percentage value of control used to establish the crosshair were 92% in the lower left quad for the double color negative control. Numbers in dot-plots indicate percentages of cells in the relevant quadrant.

D- Immunocytochemistry analysis of EphA2 on day 11 CD34+ derived DC cytospins. Staining was performed with anti-EphA2 polyclonal antibody, or with anti-EphA2 polyclonal antibody plus the peptide used for immunization (blocking peptide) as described in « Methods ». The negative control represents a polyclonal rabbit antibody of unrelated specificity. Staining was revealed using a biotinylated goat anti-rabbit IgG followed by alkaline phosphatase streptavidin.

**Figure 5 : Ex vivo isolated keratinocytes and Langerhans cells from skin expression.**

A- Immunohistochemistry analysis of EphA2 expression *in vivo*. Double staining was performed on normal serial skin sections with anti-EphA2 polyclonal antibody and the anti-DCGM4 mAb, specific for Langerin. The specificity of the staining was confirmed by the absence of detection in the presence of the
immunization peptide (data not shown). The negative control represents a rabbit polyclonal antibody and a monoclonal antibody of unrelated specificity.

B- Expression of EphA2 by *ex vivo* isolated keratinocytes from normal skin. Flow cytometry staining was performed with anti-EphA2 polyclonal antibody, or with anti-EphA2 polyclonal antibody plus the peptide used for immunization, after DC permeabilization with saponin, as described in «Methods». The negative control represents a rabbit polyclonal antibody of unrelated specificity. Results are representative of 3 experiments.

C- Expression of EphA2 by *ex vivo* isolated Langerhans cells from skin. Langerin and EphA2 expression was analyzed by flow cytometry on enriched Langerhans cells with an anti-EphA2 polyclonal antibody or with mAb DCGM4, specific for Langerin, and compared to species-specific unrelated antibodies (filled histogram). Results are representative of 3 experiments.

D- Double staining of another Langerhans cells preparation with surface FITC-CD1a, and intracellular anti-EphA2 polyclonal antibody revealed with PE-anti-rabbit IgG.

**Figure 6**: Expression of several Eph ligands in normal skin (ephrin-A3, -B1, and -B2).

A- Expression of ephrin-A3, -B1 and -B2 by day 11 CD34+-derived-DC cultured in the presence of GM-CSF and TNFα. Ephrin-A3, -B1 and -B2 expression was analyzed by cytofluorimetry with anti-ephrin-A3 (K19), anti-ephrin-B1 (C18) or anti-ephrin-B2 (P20) polyclonal antibodies directed against intracytoplasmic epitope, after DC permeabilization with saponin, as described in «Methods». Dotted-line overlay histograms show negative fluorescence control with an antibody of unrelated specificity and bold dotted-line overlay histograms represent staining after addition of the blocking peptides.

B- Immunocytochemistry analysis of ephrin-A3, -B1 and -B2 on day 11 CD34+-derived DC cytospins. Staining was performed with anti-ephrin-A3
(K19), anti-ephrin-B1 (C18) or anti-ephrin-B2 (P20) polyclonal antibodies. The specificity of the staining was confirmed by absence of detection in the presence of the immunization peptide (data not shown). The negative control represents a polyclonal antibody of unrelated specificity.

C- Immunohistochemistry analysis of ephrin-A3, -B1 and -B2 expression \textit{in vivo}. Staining was performed on normal serial skin sections with anti-ephrin-A3 (K19), anti-ephrin-B1 (C18) or anti-ephrin-B2 (P20) polyclonal antibodies. The negative control represents a polyclonal antibody of unrelated specificity (Original magnification, 200x).

\textbf{Figure 7 : Activation of Eph receptor by its ligand increases adhesion to fibronectin but not to PLL.}

A- 96 well plates were coated with serial dilution of fibronectin or PLL (from 20 to 1 $\mu$g/ml) O/N at 4°C. Non-specific binding sites were blocked at RT for 1h using 1%BSA/PBS. Day 11 CD34$^+$-derived DC (1x$10^5$ cells in 100 $\mu$l serum free medium) were plated in the presence of ephrin-A3-Fc or control Ig (10 $\mu$g/ml), and centrifuged at 1200 rpm for 3 min. After washing with PBS and fixation with 0.5% paraformaldehyde/0.5% glutaraldehyde at RT for 30 min, adherent cells were stained with 0.5% crystal violet in 20% methanol at RT for 10 min. The cells were washed 3 times with water and extracted with 50% ethanol/50mM sodium citrate pH=4.5. Values represent mean A$_{570}$ absorbances from triplicate wells. Results are representative of three independent experiments.

B-D- 96-well plates were coated with 5 $\mu$g/ml fibronectin (B) or PBS (C) or poly-L-lysine (PLL) (D), O/N at 4°C. Non-specific binding sites were blocked at RT for 1h using 1%BSA/PBS. Day 11 CD34$^+$-derived DC (1x$10^5$ cells in 100 $\mu$l serum free medium) were plated in the presence of PBS (control), Fas-Fc, ephrin-A3-Fc, ephrin-B3-Fc or ephrin-A4-Fc (10 $\mu$g/ml), and centrifuged at 1200 rpm for 3 min. After washing and fixation, adherent cells were stained with crystal violet. Values represent mean A$_{570}$ absorbances from triplicate wells.
The results comparing the different ephrin-Fc chimera are representative of three independent experiments, and overall the results with ephrin-A3-Fc are representative of 13 experiments.

**Figure 8 : β1 integrin is involved in ephrin-A3-Fc inducing DC adhesion to fibronectin.**

96-well plates were coated with 5 μg/ml fibronectin or poly-L-lysine (PLL) O/N at 4°C. Non-specific binding sites were blocked at RT for 1h using 1%BSA/PBS. Day 11 CD34+ derived DC (1x10^5 cells in 100 μl serum free medium) were plated in the presence of Ig or ephrin-A3-Fc (from 20 to 1 μg/ml), and centrifuged at 1200 rpm for 3 min. To test for the effect of the RGD peptide, cells were pre-incubated for 5 min with the peptide (10 μM) before treatment with the fusion proteins. After washing and fixation, adherent cells were stained with crystal violet. Values represent mean A570 absorbances from triplicate wells. Results are representative of three independent experiments.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Human dendritic cells express neuronal Eph receptor tyrosine kinases: role of EphA2 in regulating adhesion to fibronectin

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