The Class 6 Semaphorin SEMA6A Is Induced by Interferon- γ and Defines an Activation Status of Langerhans Cells Observed in Pathological Situations

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Originally implicated in axon guidance, semaphorins represent a large family of molecules that are now known to be expressed in the immune system. Among different semaphorins tested by reverse transcriptase-polymerase chain reaction in human immune cells, the expression of class 6 transmembrane semaphorin SEMA6A was restricted to dendritic cells (DCs). Using in-house generated monoclonal antibodies, SEMA6A expression appeared further restricted to Langerhans cells (LCs). *In vivo*, SEMA6A mRNA was expressed in freshly isolated skin LCs but SEMA6A protein was not detectable on normal skin and tonsillar epithelium. Of interest, SEMA6A protein was strongly expressed on skin and bone LCs and on LCs in draining lymph nodes from patients with LC histiocytosis or dermatopathic lymphadenitis, respectively, representing two inflammatory conditions in which LCs display an immature DC-LAMP^{low}, CD83^{low}, and CCR7⁺ phenotype. SEMA6A expression was low in resting LCs generated *in vitro* and was enhanced by interferon (IFN)- γ but not by interleukin-4, interleukin-10, IFN- α/β , or lipopolysaccharide. Most IFN- γ -induced SEMA6A-positive cells remained immature with low CD83 and DC-LAMP/CD208 expression, but they expressed CCR7 and responded to macrophage inflammatory protein-3 β (MIP-3 β /CCL19). The expression of SEMA6A, for which the ligand and function remain unknown, may therefore identify an alternative IFN- γ -dependent activation sta-

tus of LCs *in vivo*. (*Am J Pathol* 2006, 168:453–465; DOI: 10.2353/ajpath.2006.050288)

Dendritic cells (DCs) are bone marrow-derived professional antigen-presenting cells required to initiate specific T-cell immune responses.¹ Immature DCs reside in nonlymphoid tissues, similar to Langerhans cells (LCs), which form an organized network in the epidermis. During pathogen invasion, they are believed to capture antigen and, after inflammatory stimuli, leave the tissues to reach the draining lymphoid organs where they fit together in a meshwork favoring interaction with T cells. During their migration, DCs usually undergo phenotypical and functional maturation including loss of antigen capture capacity and up-regulation of co-stimulatory molecules such as CD80 and CD86. Thus, migration and organization in network constitute integral parts of DC function. Different studies have demonstrated that trafficking of DCs is controlled by soluble chemotactic factors known as chemokines,^{2–5} but other molecules are expected to play a role in the regulation of DC tissue trafficking.

Guidance of axon during the development of the nervous system involves molecules such as Slit, Robo, Eph, and semaphorins, which function by either attractive or repulsive effects through interactions with their specific receptors and regulation of cytoskeleton organization.^{6–8} In particular, semaphorins represent a large phylogenetically conserved family (>25 genes) of secreted, glycosylphosphatidylinositol-anchored and transmembrane glycoproteins that are divided in eight subclasses (subclasses 1 to 7 and V for viral).^{9,10} One characteristic of semaphorins is an N-terminal 500-amino acid extracellu-

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lar domain (called sema domain) that contains cysteine-rich repeats. Members of the semaphorin family have also been implicated in organogenesis, vascularization, angiogenesis, neuronal apoptosis, and the progression of cancer.^{11,12} The best characterized receptors for semaphorins are the plexins and neuropilins.^{6,13,14} Recently, a role for semaphorins in the immune system has emerged.^{15–17} In particular, the semaphorin SEMA4D, known as CD100, is expressed on T cells, resting B cells,¹⁸ and DCs, and it can be up-regulated after activation. Its cross-linking enhances T-cell activation and proliferation in humans.¹⁹ More recently, human and mouse sema4D have been found to enhance B cell, monocyte, and monocyte-derived DC (moDC) activation with secretion of proinflammatory cytokines and proliferation by turning off the negative signals of CD72.^{19–21} Soluble human SEMA4D can also inhibit monocyte and B-cell migration.²² The class 4 semaphorin sema4a, expressed by mouse bone marrow-derived DCs and B cells, enhances the *in vitro* activation and differentiation of T cells and the *in vivo* generation of antigen-specific T cells through binding and activation of the receptor Tim-2 (T cell conserved immunoglobulin and mucin domain), which is expressed on T cells.²³ Additionally, virus-encoded semaphorins SEMAVA/A39R and SEMAVB/AHV, and human SEMA7A/CDw108 induce aggregation of monocytes, secretion of the proinflammatory cytokines interleukin (IL)-6 and IL-8, and expression of CD54 through activation of their receptor Plexin-C1.^{13,24} These findings indicate that semaphorins not only have a role in the nervous system but are also involved in regulation of the immune system through interactions with plexins and neuropilins as well as with other receptors such as CD72 and Tim-2.¹⁷

In recent efforts to determine the expression and possible functions of cell guidance molecules on DCs, we have shown that Eph kinase receptors regulate integrin-mediated adhesion of DCs.²⁵ Herein, we have analyzed the presence of mRNA for several different semaphorins in human immune cells, in particular in DCs. We report that LCs generated *in vitro* from cord blood CD34⁺ progenitors and LCs isolated from skin preferentially expressed the class 6 semaphorin SEMA6A-1.²⁶ The class 6 semaphorins are transmembrane semaphorins composed of four members: SEMA6A, 6B, 6C, and 6D.^{26–29} Two isoforms of SEMA6A have been described: SEMA6A-1 and SEMA6A-2. The full-length SEMA6A-1 exhibits an intracellular domain that displays a small region of homology to Zyxin, with a proline-rich motif involved in the regulation of actin polymerization via binding to Ena/VASP proteins,²⁶ suggesting a role in LC trafficking or activation.

Maximal SEMA6A expression on LCs required a certain level of activation such as that provided by interferon (IFN)- γ . These cells migrated in response to CCR7 ligands but remained immature with respect to CD83 and DC-LAMP expression. *In vivo* SEMA6A protein was not detected on LCs from normal skin but was abundantly expressed on skin and bone LCs from patients with Langerhans cell histiocytosis (LCH) and on LCs in draining lymph nodes from patients with dermatopathic lymphad-

enitis (DL), which also display a phenotype similar to the one of IFN- γ -treated cells *in vitro* with respect to CD83, DC-LAMP,^{30–33} and CCR7 expression.^{30,31} The presence of SEMA6A on immature or partially mature LCs may delineate a particular pathway of LC activation *in vivo*.

Materials and Methods

Hematopoietic Factors, Cytokines, Reagents, and Cell Lines

All cultures were performed in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/L L-glutamine (all from Life Technologies, Gaithersburg, MD), and 100 μ g/ml gentamicin (Schering-Plough, Levallois-Perret, France). All hematopoietic factors and cytokines were recombinant human proteins and were used at optimal concentration as indicated: granulocyte macrophage-colony stimulating factor (100 ng/ml GM-CSF: specific activity, 2×10^6 U/mg; Schering-Plough Research Institute, Kenilworth, NJ), tumor necrosis factor (TNF)- α (2.5 ng/ml: specific activity, 2×10^7 U/mg; Genzyme, Boston, MA), stem cell factor (SCF) (25 ng/ml: specific activity, 4×10^5 U/mg; R&D Systems, Abington, UK), IL-4 (10 ng/ml specific activity, 10^7 U/mg; Schering-Plough Research Institute), IL-10 (100 ng/ml: specific activity, 10^7 U/mg; Schering-Plough Research Institute), IFN- γ (20 ng/ml: specific activity, 10^7 U/mg; R&D Systems), and transforming growth factor (TGF)- β 1 (10 ng/ml: specific activity, 5×10^7 U/mg; R&D Systems). The chemokines macrophage inflammatory protein-3 α (MIP-3 α /CCL20), MIP-3 β /CCL19, MIG/CXCL9, and IP-10/CXCL10 were from R&D Systems. In some experiments, cells were activated with 1 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) and 1 μ g/ml of ionomycin (Calbiochem, La Jolla, CA) or with 25 ng/ml of *Escherichia coli* lipopolysaccharide (LPS; Sigma-Aldrich). Murine fibroblasts transfected with human CD40 ligand (CD40L-L cells) were produced in the laboratory.²⁵

Cell Preparation

Umbilical cord blood samples, adult peripheral blood samples, and tonsils were obtained according to institutional guidelines. Peripheral blood mononuclear cells (PBMCs) were purified from human peripheral blood by Ficoll-Hypaque centrifugation. Monocytes (>95% CD14⁺) were purified from PBMCs by centrifugation over a 52% Percoll gradient followed by immunomagnetic depletion of contaminating T, B, and NK cells as described elsewhere.^{25,34} Granulocytes were purified from whole blood, T lymphocytes (>95% CD3⁺) were purified from PBMCs by immunomagnetic depletion, and B cells (>98% CD19⁺) were isolated from tonsils as previously described.³⁴ CD34⁺ hematopoietic progenitors were purified from umbilical cord blood through positive selection using anti-CD34 monoclonal antibody (mAb)-coated microbeads (Miltenyi Biotec, Bergish Gladbach, Germany) and was

achieved using Minimacs separation columns (Miltenyi Biotech) as previously described.³⁵ Epidermal cell suspensions were obtained from normal skin of patients undergoing reconstructive plastic surgery of breast or abdomen, as described elsewhere.³⁶ LC enrichment was achieved by successive density gradient centrifugation steps and depletion of basal keratinocytes. The isolated cells contained 55 to 75% CD1a⁺ LCs.

Generation of DCs from CD34⁺ Progenitors and from Monocytes

Cultures of CD34⁺ cells were established in the presence of SCF, GM-CSF, TNF- α , and 5% AB⁺ pooled human serum, as described.^{35,37} By day 6, human serum was removed, and cells were further cultured in the presence of GM-CSF and TNF- α , with or without TGF- β 1 until day 12. At this time point, aliquots of cells were activated with PMA and ionomycin for 1 and 6 hours, pooled, and then lysed for RNA extraction. For analysis of SEMA6A expression by flow cytometry, cells were collected at the time point indicated. DCs were activated by co-culture with irradiated (7500 rads) CD40L-L cells (one CD40L-L cell for five DCs) for 48 hours. In some experiments, CD1a⁺ and CD14⁺ DC precursor subsets were separated at day 6 by sequential immunomagnetic-positive selection using Minimacs separation columns (CD1a and CD14 immunobeads) (Miltenyi Biotech) and further cultured until day 10 for the CD1a⁺ DC subset in the presence of GM-CSF and TNF- α and until day 12 for the CD14⁺ DC subset in the presence of GM-CSF and TNF- α with or without TGF- β 1. moDCs were produced by culturing purified blood monocytes for 6 days in the presence of GM-CSF and IL-4. In some experiments, 5×10^5 moDCs/well (24-well culture plate) were further activated with 25 ng/ml LPS for 72 hours or by co-culture with 10^5 irradiated CD40L-L cells.

RNA Extraction, cDNA Reverse Transcription, and Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR) Analysis

Cells were lysed and total RNA was extracted by RNeasy kit (Qiagen, Valencia, CA). First-strand cDNAs were prepared after DNase I treatment (in the presence of RNase inhibitor) of 5 μ g of total RNA using oligo(dT) primers (Pharmacia, Uppsala, Sweden) and Superscript kit. Genomic contamination was excluded by quantitative PCR (Light Cycler; Bio-Rad, Hercules, CA) using human CD4 promoter probe (forward primer: 5'-TTCCACACTGGGCCACCTAT-3', reverse primer: 5'-TTGTGGGCTTACCACTGCTG-3', probe: CACTGGACACAATTGCCCTCAGG-3'). Synthesis of cDNAs was controlled by performing RT-PCR using β -actin sequence (21, 28, and 35 cycles).²⁵ RT-PCRs were performed using Ready Mix REDTaq (Sigma Chemical Co., St. Louis, MO) with the primers 5'-GTCCTGCTCCCCGCCGATTC-3' (forward) and 5'-CACTGCTGTCCCCACTTACC-3' (reverse) for SEMA4A, 5'-CAAGGGACGAGAAGGGGAAT-3' (forward) and 5'-AAAAGGGTGTACGCCAGTC-3' (reverse)

for SEMA4B, 5'-GTCCGACTGCTATGCCGAGC-3' (forward) and 5'-CGCCGATGCCAGTTGTTAAT-3' (reverse) for SEMA4C, 5'-AACGAGCCTAGTTTCGTGTTTGC-3' (forward) and 5'-GGCCTGGGTCCGGTCCACCACG-3' (reverse) for SEMA4D, 5'-CGGCACCTTTGGCTCATCTCT-3' (forward) and 5'-AAGTTCCCTCTGTGCGCCGTC-3' (reverse) for SEMA4F, and 5'-GCGTGATGTTGTCTGGCAA-3' (forward) and 5'-CACGCTCTATGTCCTGCTCA-3' (reverse) for human SEMA6A cDNA. Cycle conditions (35 cycles) were 92°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes. PCR products were cloned using the pCRIITOP vector (TA cloning kit; Invitrogen, San Diego, CA). Double-stranded plasmid was sequenced on an ABI 373A sequencer (Applied Biosystems, Foster City, CA) using dye terminator technology. Sequencher (Gene Codes, Ann Arbor, MI) software was used to analyze sequences and verify identity with human SEMA6A (accession number AF279659).

Generation of Anti-SEMA6A mAbs

Full-length SEMA6A was cloned from cDNA of total human brain (Clontech, Palo Alto, CA). Full-length SEMA6A with a C-terminal c-myc tag (intracellular domain) (SEMA6A-c-myc) was cloned into the pMET7 plasmid and was used for mouse immunization and for flow cytometric screening of mAbs. Full-length SEMA6A with an N-terminal HA tag (extracellular domain) (HA-SEMA6A) was cloned into the pDisplay (Invitrogen) plasmid and was used for immunocytochemical screening of mAbs. For the SEMA6A-c-myc construct, *SalI* restriction enzyme site (underlined) followed by the start codon was included in the forward cloning primer (5'-ATAGTCGACATGAGGTCAGAAGCCTTGCTGC-3'), and a c-myc epitope followed by the stop codon and *NotI* restriction enzyme site (underlined) (5'-ATAGCGGCCGCTTAAGATCCTCTCAGAGATGAGTTTCTGCTCCTGTACACGCATCATTGGGCT-3') was introduced in the reverse cloning primer. For the HA-SEMA6A construct, the forward primer containing *SalI* restriction enzyme site (underlined) and excluding SEMA6A signal peptide (5'-CCCGTGCGACACAGAAAGATTCTGAGCCAAT-3') (peptide signal and HA tag are included in pDisplay) and the reverse primer containing a stop codon and a *NotI* restriction enzyme site (underlined) (5'-ATAGCGGCCGCTTATGTACACGCATCATTGGGCT-3') were used. mAbs against SEMA6A were produced by immunizing BALB/c mice (Charles Rivers, Les Oncins, France) with three intraperitoneal injections of 10^6 SEMA6A-c-myc-transfected COP-5 cells in Freund's adjuvant (Sigma). Spleens were removed 3 days after a final intravenous injection of SEMA6A-c-myc-transfected COP-5, and splenocytes were fused with murine SP2 myeloma cell line using polyethylene glycol-1000 and cultured in 96-well plates using standard procedures. Hybridoma supernatants were screened for their reactivity against acetone-fixed HA-SEMA6A-transfected COP-5 cells by immunocytochemistry. Selected supernatants were confirmed by flow cytometry on SEMA6A-c-myc-transfected COP-5 cells and by immunocytochemistry. Surface and intracytoplasmic (cells were

first permeabilized with saponine) staining were performed and analyzed on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). After cloning of selected hybridomas, mAbs were produced by high-density culture INTEGRA CL 350 (IBS Integra Biosciences, Chur, Switzerland) and purified by HPLC on an anion exchange column. The mAbs 118E7 and 104B3 used in this study are of IgG1 kappa isotype and recognize the extracellular domain of SEMA6A.

Flow Cytometry Analysis

Cell-surface expression of SEMA6A was determined by flow cytometry analysis with a FACSCalibur flow cytometer (BD Biosciences). For single staining, cells were incubated for 30 minutes at 4°C with 10 μ g/ml purified anti-SEMA6A mAb 118E7 in phosphate-buffered saline, 1% human serum, washed twice, and then labeled with PE-conjugated goat anti-mouse IgG (DAKO, Glostrup, Denmark). Nonspecific staining was determined using isotype-matched control mAb. PE-conjugated anti-CD25, anti-CD83, anti-CD86, and anti-CCR7 were used for the phenotype of *in vitro*-generated DCs.

Immunohistochemistry

Biopsy samples from 12 patients referred to Necker Enfants Malades Hospital and diagnosed as having LCH³¹ or DL³⁰ were examined by immunohistochemistry. Serial cryostat sections of biopsy specimens were stained with CD1a or SEMA6A mAb 104B3 mouse primary antibodies at the appropriate concentration as determined by titration and then labeled with a goat anti-mouse peroxidase-conjugated antibody. AEC (Sigma) was used as a substrate for peroxidase. Isotype-matched antibodies were used as negative controls. Double staining was performed with double stain EnVision kit (DAKO) according to the manufacturer's instructions.

Migration Assay

Migration assays were performed using Transwell chambers (6.5-mm diameter; Costar, Cambridge, MA) with 5 $\times$ 10⁵ cells/well. CD34⁺ progenitor cell-derived DCs were incubated for 1 hour at 37°C in 5- μ m pore size inserts in the presence or the absence of MIP-3 α , MIP-3 β , IP-10, or MIG tested at 1 μ g/ml in the lower chamber. Migrating cells (in the lower chamber) were collected and counted by flow cytometry. Each experiment was performed in duplicate, and less than 10% variation was observed.

Results

Expression of Class 4 Semaphorins and SEMA6A mRNA in Cells of the Immune System

All RNA preparations were first controlled for the absence of genomic DNA contamination, and cDNA were normalized according to the β -actin PCR amplification.

Figure 1. Expression of class 4 semaphorins and SEMA6A mRNA in hematopoietic cells. RT-PCR analysis of human class 4 and 6 semaphorin expression in hematopoietic cells (**left**). cDNAs were prepared from resting and activated (1 hour and 6 hours) freshly isolated PBMCs, blood T cells, monocytes, and granulocytes or tonsil B cells. RT-PCR analysis of semaphorin expression in *in vitro*-generated CD34⁺-derived DCs and moDCs (**right**). cDNAs were prepared from cord blood CD34⁺ progenitors cultured in the presence of GM-CSF and TNF- α for 6 and 12 days, then 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), followed by 24-hour activation with CD40L-L cells, or from CD40L-L cells as control. RT-PCR was performed under standard conditions using 50 ng of cDNA for 35 cycles. All cDNA samples were normalized according to the results of human β -actin PCR amplification of 21, 28, and 35 cycles (only the amplification of 28 cycles is shown) except for CD40L-L cells normalized using mouse β -actin PCR amplification. The absence of genomic contamination was controlled in all RNA samples (before the reverse transcription step) by quantitative PCR using human CD4 promoter probe. Results are representative of RT-PCR of three independent samples.

Moreover, primers used in the study allowed us to discriminate amplification of cDNA and genomic DNA and were designed to be specific for each semaphorin without cross-reacting with any other semaphorins based on available published sequences in NCBI database. Amplified fragments were also sequenced to confirm the specificity. In a first set of experiments, RT-PCR reactions were performed with DCs derived from CD34⁺ progenitors cultured in the presence of GM-CSF and TNF- α for 12 days. cDNA for class 5 semaphorins were not amplified (data not shown), whereas mRNA for class 4 semaphorins (SEMA4A, 4B, 4C, 4D, and 4F) and class 6 SEMA6A were detected in CD34⁺-derived DCs (Figure 1). Class 3 and 7 semaphorins were not tested. In agreement with previous reports,^{17,22,38–40} SEMA4A and SEMA4D were also detected in T cells, B cells, monocytes, and granulocytes. Very faint or no band of SEMA6A mRNA could be amplified from T lymphocytes, granulocytes and PBMCs with or without activation (anti-CD3/anti-CD28 for T cells and PMA plus ionomycin for granulocytes and PBMCs). Weak SEMA6A expression was detected in B cells only after CD40L activation. Among *in vitro*-generated DCs, weak amplification of SEMA6A was detected in activated but not resting monocyte-derived DCs (moDCs), contrasting with the strong expression of SEMA4A, 4D, and 4F in DCs derived from monocytes with GM-CSF and IL-4 (Figure 1). But in CD34⁺-derived DCs, strong SEMA6A expression was detected after 12 days of culture (no signal was observed at day

Figure 2. RT-PCR analysis of human SEMA6A expression in different DC subsets generated *in vitro* and in *ex vivo*-isolated CD11c⁺ and CD11c⁻ tonsil DCs and skin LCs. **A:** Cord blood CD34⁺ progenitors were cultured in the presence of GM-CSF, SCF, TNF- α , and 2.5% AB⁺ human serum. At day 6, CD1a⁺ and CD14⁺ precursors were sorted by flow cytometry, recultured with GM-CSF and TNF- α for 6 additional days, and then activated or not as indicated. **B:** CD11c⁺ and CD11c⁻ DCs were sorted by flow cytometry from tonsil. **C:** cDNAs were prepared from basal keratinocytes and from LCs freshly isolated from normal skin activated or not with TNF- α for 48 hours. RT-PCR for SEMA6A was performed under standard conditions using 50 ng of cDNA for 35 cycles. All cDNA samples were normalized according to the results of β -actin PCR amplification and the absence of genomic contamination was controlled as described in Figure 1. Results are representative of RT-PCR of three independent samples.

6) and was maintained after CD40L activation (Figure 1). SEMA6A sequences from these DCs were analyzed and found to be identical to the full-length sequence described by Klostermann and colleagues.²⁶ All together these results show that SEMA6A is preferentially expressed in DCs, in particular in CD34⁺-derived DCs, in contrast to other tested semaphorins that are expressed on most leukocytes.

SEMA6A mRNA Is Preferentially Expressed in LCs

In vitro CD34⁺-derived DCs are composed of two subsets of DC progenitors: the CD1a⁺ subpopulation that differentiates into LCs and the CD14⁺ subpopulation that may correspond to interstitial DCs.^{35,41} To analyze SEMA6A expression, these two subpopulations of DCs were sorted by flow cytometry according to CD14 and CD1a expression after 6 days of culture of CD34⁺ progenitors and then recultured for 5 additional days with GM-CSF and TNF- α as previously described.³⁷ As shown in Figure 2A, SEMA6A mRNA was expressed at high levels in cells derived from the resting and activated CD1a⁺ subpopulation compared to that from the CD14⁺ subpopulation in

which only a weak signal was observed after CD40L activation.

In DCs isolated *ex vivo* from tonsils, no SEMA6A expression was detected by RT-PCR in CD11c⁻ DCs, and a very faint signal was detected in cDNA of CD11c⁺ cells after 35 cycles of PCR amplification (Figure 2B). In correlation with data obtained with *in vitro*-generated CD1a⁺ DCs, SEMA6A messenger was detected in both nonactivated and TNF- α -activated (48 hours) LCs isolated from skin (Figure 2C). In contrast, no SEMA6A expression was detected in basal keratinocytes. All together, these results indicate that SEMA6A is preferentially expressed in *in vitro*-generated CD1a⁺ LCs and in *ex vivo*-isolated skin LCs.

Cell Surface Expression of SEMA6A Is Restricted to the *In Vitro*-Generated LCs

To confirm the expression of SEMA6A at the protein level and to further search for its function on DCs, we generated monoclonal antibodies (mAbs) against human SEMA6A. The mAbs 118E7 (IgG1) and 104B3 (IgG1) recognizing the extracellular domain of recombinant SEMA6A were selected and used throughout this study for flow cytometry and immunocytochemistry/immunohistochemistry, respectively. Their specificity was demonstrated by flow cytometric analysis on different cell lines previously tested by RT-PCR for the presence of various semaphorins including SEMA6A (not shown). On total population of CD34⁺-derived DCs generated in the presence of GM-CSF and TNF- α at day 12, only a small fraction of cells was generally stained by the anti-SEMA6A mAb (Figure 3A, top left). In agreement with RT-PCR analysis showing preferential expression of SEMA6A mRNA in the CD1a⁺ subset (Figure 2A), flow cytometry analysis showed a clear staining of anti-SEMA6A mAb 118E7 on DCs derived from CD1a⁺ precursors but not from CD14⁺ precursors in the presence of GM-CSF and TNF- α (Figure 3A). Exogenous TGF- β 1 is required for LCs differentiation from CD14⁺ precursors or from monocytes, but it is not necessary for LC differentiation from CD1a⁺ precursors.^{35,37,42-45} Addition of TGF- β 1 from day 6 to day 12 of culture increased the detection of cell surface SEMA6A on DCs derived from total CD34⁺ or from CD14⁺ DC precursors (Figure 3B). Further activation by CD40L or LPS did not significantly up-regulate the intensity of SEMA6A expression (not shown).

In accordance with the RT-PCR results, no staining of anti-SEMA6A mAb 118E7 was detected by flow cytometry on blood T cells, B cells, NK cells, granulocytes, or monocytes, even after activation (anti-CD3/anti-CD28 for T cells and LPS plus IL-4 or IFN- γ for monocytes) (not shown). SEMA6A expression was very weakly induced on tonsil B cells cultured with CD40L and IL-4 but not with CD40L and IL-2 plus IL-10 or IFN- γ (not shown). Finally, we did not detect by flow cytometry any staining of anti-SEMA6A mAb 118E7 on blood CD11c⁺ or CD11c⁻ plasmacytoid DCs (pDCs) (not shown) or on moDCs (not shown).

Figure 3. SEMA6A expression is detected on CD1a⁺ DC subset and can be induced by TGF-β on CD14⁺ DC subset. CD34⁺ progenitors were cultured with GM-CSF, TNF-α, SCF, and 5% AB⁺ serum. At day 6, total DCs or CD1a⁺ and CD14⁺ DC subsets separated using immunomagnetic-positive selection were washed and recultured in the presence of GM-CSF and TNF-α (**A**) or GM-CSF, TNF-α, and TGF-β (**B**) until day 12. SEMA6A and Langerin expression were analyzed by flow cytometry after staining with anti-SEMA6A mAb 118E7 or anti-Langerin mAb (histograms in **solid line**) or control mouse IgG (histograms in **dotted line**) followed by PE-conjugated goat anti-mouse Ig. Histograms from one representative experiment are shown. Numbers in histograms are the mean ± SD percentage of SEMA6A-positive cells calculated from four independent experiments.

All together, these observations confirm that SEMA6A is preferentially expressed at the cell surface of *in vitro*-generated LCs and identify TGF-β as a determinant cytokine for the induction of SEMA6A expression on *in vitro*-generated LCs. However, we failed to detect cell surface SEMA6A expression on freshly isolated skin LCs by flow cytometry (not shown), despite the fact that SEMA6A mRNA was amplified in these cells (Figure 2C).

SEMA6A Is Expressed in LC-Associated Pathologies

To analyze the *in vivo* expression of SEMA6A, immunostaining with anti-SEMA6A mAb 104B3 was performed on sections of organs containing CD1a⁺ LCs. No staining was observed in normal skin (10 of 10) (Figure 4A) or in tonsils (that also contain mature DCs) (not shown), even with sensitive amplification technologies. We further analyzed SEMA6A expression on LCs in pathological samples from inflammatory LCH and DL. Interestingly, SEMA6A was abundantly expressed in cutaneous (Figure 4B), bone (Figure 4C), and lymph node LCH in eight of eight patients. The strong expression of SEMA6A completely correlated with that of the CD1a marker on serial sections and was confirmed by double staining, as shown in Figure 4C (panel i) in bone lesion. CD1a expression showed that these SEMA6A-positive cells were LCs. Moreover, these DCs expressed CD68 and the LC-specific lectin Langerin (CD207) but not the maturation markers CD83 and DC-LAMP (not shown).³¹ The aggre-

gates of cells expressing high levels of SEMA6A in cutaneous LCH were localized in the dermis (Figure 4A, e and f). We also observed co-expression between CD1a and SEMA6A on LCs accumulating in draining lymph nodes (LNs) from three of three patients with DL (Figure 5).³⁰ In DL, LCs are recruited in high numbers in the sinus and T-cell area of inflammatory skin-draining LNs. These LCs expressed CD1a and displayed typical morphology of LCs (Figure 5, c and d), and have previously been shown to maintain a peculiar activated yet immature phenotype because most of these LCs express Langerin, CCR7, and CD68 but do not express CD83 and DC-LAMP maturation markers.³⁰

IFN-γ Up-Regulates SEMA6A Expression on DCs

To further determine which factors up-regulated SEMA6A expression in LCs, we tested the effects of proinflammatory cytokines on SEMA6A expression on *in vitro*-generated LCs (CD34⁺-derived DCs cultured as described above in the presence of GM-CSF, TNF-α, and TGF-β from day 6 to day 10 and then cultured for 48 hours with different cytokines). Among factors tested [TNF-α, LPS (TLR-4 ligand), R-848 (TLR-8 ligand), poly I:C (TLR-3 ligand), IL-1β, IL-2, IL-3, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, CpG 2216 (TLR-9 ligand), IFN-γ, and type I IFN-α/β] (data not shown), only IFN-γ showed a significant effect on SEMA6A expression. Indeed, as shown in Figure 6A,

Figure 4. LCs in LCH strongly express SEMA6A. Serial sections from normal skin (**A**), skin lesions (cutaneous LCH) (**B**), and bone lesions (eosinophilic granuloma of LCH) (**C**) were stained with CD1a (**a, c, e, g**) or SEMA6A mAb 104B3 (**b, d, f, h**). **i:** Double staining for CD1a (in blue) and SEMA6A (in red) was performed in bone lesion. Staining was detected by peroxidase-conjugated goat anti-mouse antibody using AEC as peroxidase substrate. For the double staining, mAbs were detected using double-stain EnVision kit. Original magnifications: $\times 10$ (**a, b, g, h**); $\times 2.5$ (**c, d**); $\times 40$ (**e, f**); $\times 100$ (**i**). Data are observed in 8 of 8 patients with LCH and 10 of 10 normal skin samples.

IFN- γ increased cell surface SEMA6A expression from 5% in GM-CSF to 57% in GM-CSF plus IFN- γ (compare to 25% in GM-CSF plus TNF- α plus TGF- β ; Figure 3B).

In these experimental conditions, the low SEMA6A expression after treatment with GM-CSF alone (Figure 6A) was attributable to the absence of TGF- β from day

Figure 5. Expression of SEMA6A on LCs in lymph nodes from DL. Serial sections from draining lymph node of DL were stained with CD1a (**a, c**) or SEMA6A mAb 104B3 (**b, d**). F denotes a B-cell follicle in **c** and **d**. **c** and **d** are higher magnifications of the boxes in **a** and **b**, respectively. Staining was detected by peroxidase-conjugated goat anti-mouse antibody using AEC as peroxidase substrate. Data are observed in three of three patients with DL. Original magnifications: $\times 2.5$ (**a, b**); $\times 40$ (**c, d**).

10 to 12. This effect required a first culture with TGF- β (days 6 to 10) because no SEMA6A staining was observed when CD34⁺ cells were kept in culture with GM-CSF plus TNF- α alone before IFN- γ addition (Figure 6B). The up-regulation of surface SEMA6A expression by IFN- γ was correlated with an increased level of SEMA6A mRNA in IFN- γ -activated DCs (Figure 6C). Moreover, IL-10 and IL-4, known to inhibit IFN- γ activities, reduced the intensity of SEMA6A staining induced in the presence of IFN- γ (Figure 6A). Of note, SEMA6A expression was not up-regulated by LPS and was even down-regulated by LPS in IFN- γ -pretreated DCs (Figure 6A). In contrast to DCs derived from CD34⁺ progenitors, IFN- γ did not induce SEMA6A expression on moDCs or *ex vivo* isolated blood DC populations (not shown). Finally, although a faint signal was detected by RT-PCR in CD40L-activated moDCs, no expression could be detected by flow cytometry on CD40L- or LPS-activated moDCs (not shown).

Based on the selectivity of the IFN- γ effect on SEMA6A expression, we assessed by immunohistochemistry the expression of IFN- γ and IFN- γ R1 on LCH biopsies. Although we could not detect significant IFN- γ expression, a strong expression of the IFN- γ R1 was observed

on LCH cells (Figure 7), contrasting with the low expression on resident skin LCs (Figure 7).

In Vitro Generated LCs Expressing SEMA6A Display an Immature Phenotype but Migrate in Response to MIP-3 β

IFN- γ up-regulated SEMA6A expression while activators such as LPS down-regulated IFN- γ -induced SEMA6A expression. Because DCs in LCH and DL express SEMA6A and have been shown to possess a CD83^{low} DCLAMP^{low} immature phenotype, we investigated the expression of maturation markers such as DC-LAMP/CD208, CD86, and CD83 on LCs generated *in vitro* with GM-CSF, TNF- α , TGF- β , and IFN- γ as described above. Interestingly, *in vitro*-generated LCs, activated by IFN- γ and expressing SEMA6A, remained immature with an absence of CD83, CD86, DC-LAMP, and CD25 markers but matured normally in response to LPS activation, although lacking SEMA6A expression (Figure 8A).

However, in agreement with a previous report,⁴⁶ IFN- γ -stimulated LCs expressed CCR7 (Figure 8A) and responded to the CCR7 ligand MIP-3 β /CCL19 with a mi-

Figure 6. IFN- γ and TGF- β up-regulate SEMA6A expression on CD34 $^{+}$ progenitor-derived DCs. **A–C:** DCs derived from CD34 $^{+}$ progenitors were cultured in the presence of GM-CSF, TNF- α , SCF, and 5% AB $^{+}$ serum. At day 6, DCs were washed and cultured in the presence of GM-CSF, TNF- α , and TGF- β (**A**, **C**) or with GM-CSF and TNF- α (**B**). At day 10, DCs were washed and cultured with GM-CSF with or without IFN- γ (20 ng/ml) (**A–C**) and with or without IL-4 (10 ng/ml), IL-10 (100 ng/ml), or LPS (25 ng/ml) for 2 days (**A**). SEMA6A expression was analyzed by flow cytometry after staining of cells with anti-SEMA6A mAb 118E7 (histograms in **solid line**) or control mouse IgG (histograms in **dotted line**) followed by PE-conjugated goat anti-mouse antibody. **C:** SEMA6A expression was also analyzed by RT-PCR as described in Figure 1. cDNA were normalized by quantitative PCR using β -actin probe, and the absence of genomic contamination was controlled as described in Figure 1. Data presented are representative of three independent experiments.

gration index of 10, whereas they did not migrate in response to the CCR6 ligand MIP-3 α /CCL20 or other chemokines such as MIG/CXCL9 or IP-10/CXCL10 (Figure 8B). These observations suggest that SEMA6A expression delineates a discrete stage of CCR7 $^{+}$ immature LCs.

Discussion

The expression of molecules primarily known for their function in the nervous system has recently been shown in the immune system. SEMA6A, described in the present study as selectively expressed on DCs, is a new example of such a molecule. Among the different semaphorins analyzed, SEMA6A appears to be the most DC-restricted member of the family. Other semaphorins expressed in DCs such as SEMA4A and 4D are also present in T and B lymphocytes, granulocytes, and monocytes, as previously reported,^{22,23,38–40} and we also detected a broad expression of other semaphorin mRNAs (including SEMA4B and 4F) by lymphocytes and myeloid cells. In contrast, mRNAs for other semaphorins such as SEMA5A were not detected by RT-PCR analysis in DCs (data not shown). Beside semaphorins, other molecules involved in

axon guidance have been reported to be expressed in the immune system. In particular, neuropilin-1 has been shown to be expressed on DCs⁴⁷ and Robo/Slit by T cells⁴⁸; we also recently reported the expression of several Eph proteins on DCs.^{25,49}

Within the different subsets of DCs, SEMA6A expression is restricted to LCs, as demonstrated by 1) expression of the protein on the LC progeny of CD34-derived CD1a $^{+}$ precursors (spontaneous differentiation); 2) expression on the TGF- β -driven LC progeny of CD34 $^{+}$ -derived CD14 $^{+}$ precursors; 3) detection of the mRNA in LCs isolated from skin; 4) high levels of protein expression on LCs from patients with cutaneous and bone LCH and with DL; and 5) absence of mRNA or protein in blood circulating DC subsets and moDCs. Such restricted expression of SEMA6A argues for a role related to specific LC functions that remain primarily under-explored. The principal characteristic of LCs is their status of resident cells with a very low turnover within pluri-stratified epithelia such as in the skin,⁵⁰ where they form a network with a tightly controlled density. Based on the role of semaphorins in the nervous system that control the directional growth of axons through attractive or repulsive cues,^{7,51} it can be hypothesized that SEMA6A may play a role in the epidermal LC network.

In normal conditions, we could not detect the protein on skin LCs either by flow cytometry or by immunohistology. However, SEMA6A mRNA is up-regulated on TNF- α activation in skin LCs and the expression of SEMA6A on *in vitro*-generated LCs is strongly up-regulated on IFN- γ treatment. Furthermore, IFN- γ -stimulated *in vitro*-generated LCs that express SEMA6A did not co-express DC-LAMP and CD83, markers of mature DCs and express CCR7 and responded to the chemokine MIP-3 β but not to MIP-3 α . MIP-3 β /CCL19 has been described to induce migration through CCR7 of mature/activated DCs, while MIP-3 α /CCL20 induces migration of immature DCs through CCR6.⁵² These data suggest that these IFN- γ -stimulated LCs were partially activated but not fully mature. SEMA6A expression was only induced by IFN- γ and was down-regulated by all other tested cytokines or activators, such as LPS. Furthermore, SEMA6A expression was never detected in tonsil, suggesting that mature DCs present in the tonsils either down-regulated or did not up-regulate SEMA6A expression. This suggests that SEMA6A up-regulation on LCs was dependent on a particular activation pathway produced by IFN- γ . Importantly, high levels of SEMA6A expression were detected in LCs present in cutaneous and bone (eosinophilic granuloma) lesions from LCH patients and in lymph nodes from DL patients. Expression of SEMA6A completely correlated with the LC markers CD1a and Langerin.³¹ These LCs are activated, but immature as evidenced by the absence of the maturation markers DC-LAMP and CD83,^{30,31,33} in agreement with our *in vitro* results. LCs from LCH and DL can express the chemokine receptor CCR7 (data not shown),^{30,53} suggesting that these LCs could respond to MIP-3 β /CCL19 as observed in our *in vitro* differentiation model. MIP-3 β /CCL19 responsiveness and CCR7 expression are restricted to activated DCs^{52,54,55} and have no chemotactic activity on imma-

ture DCs.⁵⁵ Furthermore, in LCH lesions, IFN-γ receptor (chain 1) was up-regulated compared to normal skin as detected by immunohistochemistry (Figure 7), suggesting an involvement of IFN-γ in this pathology. We could hypothesize that in these pathologies, LCs are activated by an alternative pathway, such as IFN-γ, leading to an intermediate CCR7⁺ immature state.

Induced expression on activation argues against a role of SEMA6A in controlling the LC network during homeostatic conditions. However, the induction of SEMA6A expression at the cell surface during inflammation

may trigger repulsive signals between epithelial cells and LCs, contributing together with CCR7 ligand responses^{2,55} to the first step of emigration out of the epidermis. The expression of SEMA6A on immature LCs in LCH and DL, accumulating in dense aggregates at distance of the epidermis (dermis, bone, lymph node), may argue in favor of this hypothesis. Moreover, Klostermann and colleagues²⁶ have shown that SEMA6A contains, in its intracellular domain, motifs of interaction with enabled/vasodilator-stimulated phosphoprotein-like protein (EVL), a proline-rich protein of the Ena/VASP family im-

A **B**

Figure 8. IFN- γ -stimulated *in vitro*-generated LCs expressing SEMA6A maintain an immature phenotype but migrate in response to MIP-3 β . Cord blood CD34⁺ progenitors were cultured until day 6 in the presence of GM-CSF, TNF- α , SCF, and 5% AB⁺ serum, washed, and cultured with GM-CSF, TNF- α , and TGF- β . **A:** At day 10, DCs were washed and cultured for 2 days with GM-CSF or GM-CSF and LPS or GM-CSF and IFN- γ . Phenotype of DCs in these three different activation conditions was determined by flow cytometry using anti-CD25, anti-CD83, anti-CD86, anti-DC-LAMP, anti-CCR7, and anti-SEMA6A 106C3 Ab. **B:** At day 10, CD34⁺ DCs were washed and cultured for 2 days with GM-CSF plus IFN- γ . Migration assay was performed in Transwell chambers. Chemokines were present in the lower chamber (1 μ g/ml), and 5×10^5 DCs were seeded in the upper chamber. After 1 hour, migrating cells in the lower chamber were collected and counted by flow cytometry. Results are representative of three independent experiments.

plicated in filament dynamic control and actin-based motility.^{26,56,57} Thus, SEMA6A may participate in the control of LC emigration through the regulation of the level of actin polymerization and modulation of cytoskeleton rearrangement that are involved in all steps of DC migration.

Alternatively, SEMA6A may function as a ligand engaging a cell surface receptor as described for other semaphorin members. Recently, Plexin A1 was identified as the major SEMA6D-binding receptor,⁵⁸ but the ligand/receptor of other SEMA6 are still unknown. It would be of interest to understand the function of SEMA6A expressed on these activated yet immature LCs and its potential role in regulation of immune responses such as the development of T-cell responses. Of importance, DCs with an immature phenotype have been suggested to induce or maintain immune tolerance,^{1,59–61} and SEMA6A may engage a counter structure expressed on T cells contributing to the modulation of T-cell function. In this context, in

a mouse model, IFN- γ was shown to block the capacity of LCs pulsed with tumor-associated antigens, to elicit anti-tumor immunity, showing not only the alternative activation status but also the inhibition of the immune response⁶² as suggested here in the human LCH.

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