Neuronal Repellent Slit2 Inhibits Dendritic Cell Migration and the Development of Immune Responses[1]

Hongbing Guan,* Guorui Zu,* Yi Xie,† Hao Tang,§ Martin Johnson,‡ Xiaochun Xu,‖ Christophe Kevil,# Wen-Cheng Xiong,† Craig Elmets,* Yi Rao,¶ Jane Y. Wu,§ and Hui Xu[2]*

One of the essential functions of dendritic cells is to take up Ags in peripheral tissues and migrate into secondary lymphoid organs to present Ags to lymphocytes for the induction of immune responses. Although many studies have demonstrated that the migration of dendritic cells is closely associated with the development of immune responses, little is known about factors that inhibit dendritic cell migration and control the extent of immune responses to Ag stimulation. We show that Slit2, a neuronal repellent factor, is up-regulated in the skin by allergen sensitization and down-regulates the migration of Langerhans cells. The effect is mediated by direct interaction of Slit2 with cells that express a Slit-specific receptor, Robo1. Slit2-mediated inhibition of Langerhans cell migration results in suppression of contact hypersensitivity responses. These findings provide insights into a novel mechanism by which Slit2 functions as an anti-inflammatory factor for the initiation of immune responses. ***The Journal of Immunology,*** **2003, 171: 6519–6526.**

Dendritic cells (DC)[3] play a crucial role in the initiation and regulation of adaptive immune responses (1). These multifunctional cells possess several attributes that enable them to present Ag effectively, one of which is their ability to migrate into peripheral tissues and, after Ag uptake, to home to the secondary lymphoid organs. During transit from the peripheral tissues to the secondary lymphoid organs, the DC undergo maturation and become potent APCs that are capable of inducing immune responses. This migration of DC from the site of infection or inflammation to the secondary lymphoid organs is critical to the initiation of adaptive immune responses (2, 3). The mechanisms underlying the migratory process include directional motility and adhesion, as well as rolling and extravasation, which are regulated by an assortment of chemokines, cytokines, and adhesion molecules (4–8). Although many studies have focused on the identification and characterization of factors that can stimulate the migration of DC (6, 9), little is known concerning factors that can down-regulate the process.

The down-regulation of the migration of DC is of particular interest in the search for strategies to control hypersensitivity reactions. Contact hypersensitivity (CHS) is a T cell-mediated delayed-type immune response that is induced by allergens exposed to the skin. A subpopulation of immature DC, the Langerhans cells, are found in the suprabasal layer of the epidermis, permitting the rapid initiation of immune responses to the innumerable Ags and allergens that are encountered by the skin. It has been shown in animal models of CHS that, after sensitization by hapten, the hapten-carrying Langerhans cells migrate out of the epidermis and home to the regional lymph nodes where they activate hapten-specific T cells (10, 11). Hapten sensitization induces the production or up-regulation of the various cytokines, chemokines, and adhesion molecules that are involved in the migration of Langerhans cells from the skin to the regional lymph nodes (12–15). Manipulation of Langerhans cell migration has been shown to enhance or inhibit the CHS response (16–19). It is reasonable, therefore, to speculate that down-regulation of the migration of Langerhans cell migration might result in amelioration of the CHS responses to hapten sensitization.

Little is known about factors that are able to inhibit the migration of Langerhans cells in the skin and regulate allergen-induced contact hypersensitivity. Recently, it has been demonstrated that the Slit proteins are able to inhibit chemokine-mediated leukocyte chemotaxis, suggesting that they may act as inhibitory factors in the regulation of inflammatory reactions (20). Slit proteins are repulsive factors that control axon guidance and neuronal migration (21–23). This family of secreted proteins, encoded by the *slit* genes, has been identified in several species, including humans and mice (24, 25), with three isoforms (Slit1, 2, and 3) having been described in mice. Although the expression of Slit1 is restricted to the nervous system, Slit2 and Slit3 are expressed in other tissues (20, 26). The receptors of Slits are the transmembrane Robo (roundabout) proteins, which are expressed by leukocytes as well as neurons (20, 27). It has not yet been determined whether Slits play a role in the regulation of leukocyte migration in vivo or in the development of immune responses.

In the present study, we used recombinant Slit2 and a soluble form of the Robo receptor (RoboN) to determine whether Slits can inhibit the migration of Langerhans cells and prevent the delayed-type hypersensitivity responses induced by contact allergens in the skin. Through the use of in vitro assays, we show that Slit2 can inhibit the migration of Langerhans cells and that this effect can be blocked by treatment with RoboN. Through the use of in vivo

Departments of *Dermatology, †Pathology, and ‡Toxicology and Pharmacology, University of Alabama, Birmingham, AL 35294; Departments of §Pediatrics and Molecular Biology and Pharmacology and ¶Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, MO 63110; ‖Department of Clinical Cancer Prevention, University Texas M. D. Anderson Center, Houston, TX 77030; and #Department of Pathology, Louisiana State University Health Sciences Center, Shreveport, LA 71130

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[2] Address correspondence and reprint requests to Dr. Hui Xu, Department of Dermatology, University of Alabama, VH 566B, 1670 University Boulevard, Birmingham, AL 35294. E-mail address: xuhui@uab.edu

[3] Abbreviations used in this paper: DC, dendritic cells; CHS, contact hypersensitivity; Robos, round about proteins; DNFB, dinitrofluorobenzene.

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assays, we were able to demonstrate that exogenous application of Slit2 both inhibits the migration of Langerhans cell after epicutaneous application of hapten and suppresses the initiation of contact hypersensitivity responses. In conjunction with the expression of Slit2 in the skin, these data suggest a previously undescribed regulatory mechanism, which may contribute to the control of immune responses, either by inhibiting their initiation or curtailing their magnitude and duration.

Materials and Methods

Reagents and animals

C57BL/6 mice were purchased from Charles River Laboratories (Boston, MA), and A/J mice were from Harlan (Indianapolis, IN). Female mice 6–8 wk of age were used in the experiments. A/J mice were used for the experiments with the Langerhans cell line XS106, and the rest of experiments were conducted with C57VL/6 mice. All animal procedures were performed according to National Institutes of Health guidelines under protocols approved by the Animal Care and Use Committee of the University of Alabama (Birmingham, AL).

The hybridoma 2.4G2, which is specific for CD16/32, was purchased from the American Type Culture Collection (Manassas, VA). The Abs were purified from the culture supernatants by affinity chromatography using a protein G-coupled Sepharose column (Gamma-Bind Plus; Pharmacia, Uppsala, Sweden). PE- or FITC-labeled anti-mouse Ia/IE, CD11b, and isotype-matched control Abs were purchased from BD PharMingen (San Diego, CA). GM-CSF and IL-4 were purchased from Sigma-Aldrich (St. Louis, MO), and TNF-α was from Genzyme (Cambridge, MA). Dinitrofluorobenzene (DNFB) and oxazolone were purchased from Sigma-Aldrich.

A panel of chicken anti-rat Robo1 Abs was generated against synthetic Robo1 peptides by Aves Labs (Tigard, OR). One of the Abs (2855), which was raised against the peptide corresponding to Robo1 aa 106–119, specifically stained RoboN-transfected 293 cells. The sequence of the peptide is shared by both mouse and rat Robo1. Cy3-labeled anti-chicken Ab was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

The HEK293 cells that produce Slit2 proteins tagged with c-*myc* or the extracellular domain of rat Robo1 tagged with an hemagglutinin A tag (RoboN) have been described (20, 28). The cells were cultured in DMEM with 5% FCS. Slit2 and RoboN were partially purified from the supernatants as described previously. The supernatant from parental HEK cells was used as controls.

The mouse Langerhans cell line XS106 was kindly provided by Dr. A. Takashima (University of Texas Southwestern Medical School, Dallas, TX). These cells express a mature Langerhans cell phenotype and were maintained in culture as described previously (29).

Skin explant culture

Skin explant culture of hapten-treated skin was conducted as described previously (30). Briefly, the dorsal side of mouse ears was painted with 5 μl of 0.5% DNFB and harvested 2 h later. In some experiments, 3% oxazolone was used. The hapten-treated dorsal skin was peeled off and placed on the top of DMEM with 10% FCS in 24-well tissue culture plates with one ear per well. Purified Slit2 or RoboN was added into the culture to detect the effect on cell migration. In some experiments, Slit2-tranfected or parental HEK293 cell-conditioned media were applied. The migratory cells were harvested and counted at the indicated times after cultures.

In vivo DC migration assay

Bone marrow-derived DC and the Langerhans cell line, XS106, were used to detect the migration of DC in vivo as described (30). Bone marrow-derived DC were generated from cultures of bone marrow cells in the presence of GM-CSF and IL-4 as described previously (30). In the experiments, the DC were treated with Slit2-transfected or parental HEK293 cell-conditioned culture supernatants at 37°C for 2 h and labeled with ^{51}Cr (Amersham). About 1 million cells (5–10 $\times$ 10^5 cpm) were injected into the left footpad of mice anesthetized with pentobarbital (50–60 mg/kg). The draining popliteal lymph node, the tissue around the lymph node and the injected foot were collected 16–18 h later and processed for measurement of the isotope (cpm) in a gamma counter (Wallac, Boston, MA). The migration index was calculated according to the formula: Migration index = [(cpm of LN − cpm of surrounding tissues)/cpm of injected foot]. To determine whether the treatment with Slit2 caused any toxic effect on the cells, XS106 cells were treated with Slit2 in the same way as for the in vivo migration assay and placed in culture at graded concentrations for 48 h. The proliferation of the cells was measured by [^{3}H]thymidine incorporation for the last 18 h.

Elicitation of contact hypersensitivity

Purified Slit2 (1.5 nM in 30 μl of PBS), RoboN (3 nM in 30 μl of PBS) or PBS was injected s.c. into the dorsal side of one ear. The injected dorsal side ear skin was painted with 10 μl of 3% oxazolone 2 h later. Mice without injection in the ear served as positive controls. The other ear of the mice was challenged with 10 μl of 1% oxazolone 5 days after sensitization, and ear swelling was assessed 24 h later with a micrometer. Naive mice that were not sensitized but were challenged served as negative controls. Four mice were used per group and the experiment was repeated 3 times.

Staining of epidermal Langerhans cells

The preparation and staining of epidermis were performed as published previously with minor modifications (31). In this study, purified Slit2, RoboN, or PBS was injected s.c. into the dorsal side of ears. Two hours later, the injected side of ear was painted with 10 μl of 3% oxazolone. The ears were harvested 18–20 h after hapten treatment, and the epidermal sheets were separated after incubation in 0.5 M ammonium thiocyanate at 37°C for 20 min. To stain Langerhans cells in the epidermal sheets from skin explant cultures, skin explants were harvested 48 h after the cultures and epidermal sheets were prepared.

PE-labeled anti-Ia/IE Ab was applied to stain Langerhans cells in epidermal sheets, using PE-labeled isotype-matched nonspecific Abs as controls (30). Three images of each epidermal sheet were captured with a digital camera ($\times$20 objective; AxioCam; Zeiss, Oberkochen, Germany) connected to a Zeiss fluorescence microscope, and the images were evaluated using Photoshop software (Adobe Systems, San Jose, CA). The number of positive cells was counted on each image, and the mean number of positive cells per square millimeter was calculated for each experimental group, with 3 mice per group (18 images of 6 ears). The experiment was repeated three times.

RT-PCR analysis

The expression of Slit mRNA in the skin was examined by RT-PCR. Briefly, mice were sensitized once on the shaved abdominal skin with 3% oxazolone, and the hapten-treated skin was harvested at 4, 24, or 48 h after sensitization (32). Naive skin without any treatment served as control. Total RNA was extracted using Trizol (Life Technologies, Gaithersburg, MD) according to the manufacturer's instruction. First-strand DNA was synthesized using an Omniscript Reverse Transcriptase kit (Qiagen, Valencia, CA) according to the instructions. The DNA was amplied using the PLATINUM.PCR Supermix kit (Life Technologies). The primers for mouse Slit1, Slit2, and Slit3 were designed according to the sequences of Slit cDNA from the National Center for Biotechnology Information GenBank (Table I). Reaction mixtures were subjected to the following amplification protocol: 1 cycle at 94°C for 4 min; and 30 cycles at 94°C for 45 s,

Table I. *Information of primers applied for determination of Slit mRNA expression by RT-PCR and real time RT-PCR*

Primers	Sequence (5′–3′)	PCR Product (bp)
For RT-PCR		
Slit1	cga ctg agc tac gac tga ac gaa cat gcc act tct aat gga c	203
Slit2	tta cgc tgc ctg tca aac ctt cac cac ttt ctc aac ctc	163
Slit3	aag cga ata gac atc agc aag cat aca gag aga gca gat tgag	235
GAPDH	aat ggt gaa ggt cgg tgt gaa c gaa gat ggt gat ggg ctt cc	225
For real time RT-PCR		
Slit2 primer	agtcggaatataaggaacctggaagagtagtgagtaataatttatctgccatttct	
TaqMan probe	cacgctgtgccggccctg	

58°C for 30 s, and 72°C for 60 s. PCR products were separated by agarose gel electrophoresis and the results analyzed using a gel documentation system (BioRad, Hercules, CA). As controls, RNA samples without reverse transcription were subject to PCR to exclude DNA contamination.

To quantitatively detect the expression of Slit2 in the skin, real time RT-PCR was performed as described previously (33, 34). Oligonucleotides designed using software from PerkinElmer (Wellesley, MA) were synthesized as shown in Table I and purified by HPLC (PerkinElmer) after synthesis. Total RNA was diluted to a final concentration of 10 ng/μl with RNase-free water containing 12.5 μg/ml total yeast RNA as a carrier. The amplification reactions were performed in 1× TaqMan Buffer A in a final volume of 25 μl. Final Slit2 forward, reverse, and probe concentrations were 300, 200, and 200 nM, respectively. Thermal cycling conditions were 30 min at 48°C followed by 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. The relative standard curve method was used to estimate Slit2 expression (33). All PCR were run in triplicate, and reactions with correlation coefficients falling below 0.98 were repeated. Control reactions confirmed that no amplification occurred when yeast total RNA was used as a template or when no-template-control reactions were performed (data not shown).

In situ hybridization

To detect the distribution of Slit2 in the skin, nonradioactive in situ hybridization was performed as described previously (35). Briefly, ear skin from naive mice and mice that were sensitized with oxazolone for 24 h were harvested and fixed in 10% buffered formalin for 24 h. The skin samples were embedded in paraffin and cut into 4-μm sections. Mouse Slit2 cDNA fragment between 955 and 1685 bp was cloned into pGEM vector (Promega, Madison, WI). The plasmid was linearized by *Apa*I, and digoxigenin-labeled sense and antisense cRNA were generated by using SP6 polymerase in the presence of digoxigenin-UTP. Hybridization and detection of the probe were conducted as described (35).

Immunocytochemical staining with anti-Robo1 Ab

The anti-Robo1 Ab (2855) was applied to stain HEK293 and HEK293/RoboN, Langerhans cell line XS106, bone marrow-derived DC and migratory cells from skin explant cultures. HEK293 and 293/RoboN cells were treated with Golgi Stop (BD PharMingen, San Diego, CA) for 6 h to increase the intracellular signal of RoboN proteins. Slides were made with 2–4 × 10^4 cells/slide in a Cytospin centrifuge (Thermo Shandon, Pittsburgh, PA). Cells were fixed in cold acetone for 10 min and rehydrated in PBS. Nonspecific binding was blocked by incubating slides with 10% PBS plus 4 μg of 2.4G2 Ab (rat anti-mouse CD16/CD32). Slides were then incubated with purified chicken anti-Robo1 Ab 2855 (2 μg/slide) at room temperature for 30 min. Normal chicken serum was used as a negative control. Following three washes, the slides were incubated with Cy3-labeled anti-chicken Ab (1/2000) at room temperature for 30 min. Following three washes, the slides were stained with DAPI and mounted.

To determine the specificity of the anti-Robo1 Ab, purified recombinant RoboN was applied to absorb the Ab. Ab solution (2 μg) was incubated with purified RoboN (0.2 nM) at room temperature for 30 min and then applied on samples. To characterize Robo1-positive migratory cells from skin explant cultures, FITC-labeled rat anti-mouse Ia/IE or CD11b Ab was applied (0.2 μg/slide) to double-stain cells.

Statistical analysis

The differences between experimental groups were analyzed using Student's t test with $p < 0.05$ being considered as statistically significant.

Results

Slit2 inhibits hapten-induced migration of Langerhans cells

Epicutaneous application of haptens induces the emigration of Langerhans cells from the skin. We have shown previously that hapten treatment stimulates the migration of Langerhans cells from mouse ear skin in explant cultures, with the Langerhans cells representing ~60–70% of the total migratory cells in these cultures. The Langerhans cells that migrate from hapten-treated skin can be used successfully in adoptive transfer of CHS responses into naive animals (30). To determine whether Slit proteins can inhibit hapten-induced Langerhans cell migration, mouse ear skin was treated with a hapten, DNFB, and then cultured as skin explants in the presence or absence of recombinant mouse Slit2. The number of migratory cells in the culture medium containing Slit2 was clearly lower (3- to 4-fold) than that of the untreated controls (Fig. 1*A*) at 48 h after initiation of the culture. The level of cell migration in the control, HEK293-conditioned supernatants was comparable with that observed in the fresh medium (DMEM plus 5% FCS). Additional experiments demonstrated that in the presence of Slit2, Langerhans cells were arrested in the skin. The number of Ia/IE-positive Langerhans cells in the epidermis of the skin explants from Slit2-containing cultures was significantly higher than those from the control cultures (Fig. 1*B*). This result also suggests that the inhibitory effect of Slit2 on Langerhans cell migration in the explant cultures may not result from cell death caused by toxic effect. This issue will be further confirmed in the following experiments.

Quantitation of the migratory Langerhans cells in cultures containing different concentrations of Slit2 at 48 h after initiation of cultures confirmed that the inhibitory effect of Slit2 on the migration of Langerhans cells was dose dependent (Fig. 1*C*). At the highest concentration used (10 nM), the inhibition of the migration was 4-fold greater than that observed in the controls.

In the absence of Slit2, the numbers of the migratory Langerhans cells in the explant cultures peaked at 72 h. Thereafter, the Langerhans cells died rapidly, and the cell number in the supernatant declined (data not shown). In the presence of purified Slit2 (10 nM), the numbers of migratory Langerhans cells in the explant cultures did not exhibit a significant increase at any time during the culture and was 5-fold lower than the numbers in the control culture at 72 h (Fig. 1*D*).

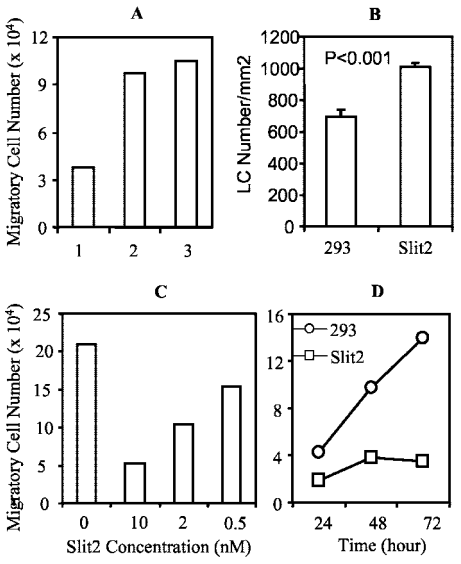

FIGURE 1. Recombinant Slit2 inhibits the hapten-induced migration of Langerhans cells in skin explant culture. Mouse ear skin was treated with hapten (DNFB) before explant culture in medium containing Slit2 or conditioned medium from the parental HEK293 cells. *A*, At 48 h after initiation of culture, the migration of Langerhans cells was inhibited in culture medium containing Slit2 (10 nM) (Column 1) compared with migration in HEK293-conditioned or fresh medium (Columns 2 and 3) (four ear explants per group). *B*, The number of Langerhans cells in the epidermis of skin explants (6 per group) was detected by immunohistochemical staining with anti-Ia/IE Ab. The difference between two groups is significant ($p < 0.01$). *C*, Analysis at 48 h after initiation of culture showed that the inhibitory effect of Slit2 on Langerhans cell migration was dose dependent (eight ear explants per group). *D*, The time course of the effects of Slit2 (10 nM) on Langerhans cell migration showed a peak at 48 h (four ear explants per group). Data represent the total number of migratory cells in each group and are representative of two to three repeated experiments.

The effect of Slit2 on Langerhans cell migration is neutralized by a soluble form of the receptor, RoboN

To determine the specificity of the inhibitory effect of Slit2 on the migration of Langerhans cells, a soluble Robo receptor was added into the explant cultures to neutralize Slit2 before culture with hapten-treated ear skin. The soluble receptor, RoboN, which contains the extracellular domain of the molecule, was described previously (20, 28). We found that the inhibitory effect of Slit (2 nM) on Langerhans cell migration was neutralized by RoboN in a dose-dependent manner (Fig. 2*A*). At the highest concentration of RoboN used (10 nM), the migration of the Langerhans cells approximated that observed in the skin that had not been treated with Slit2. Additional experiments exhibited that addition of RoboN (5 nM) in the explant cultures increased the migration of Langerhans cells in the absence of exogenous Slit2, implicating that neutralization of endogenous Slits can enhance DC migration after hapten sensitization (Fig. 2*B*). These results indicate that the inhibitory effect of Slit2 on hapten-induced migration of Langerhans cells from the skin is specific and appears to be mediated by the Robo receptors.

Slits directly interact with DC

Hapten sensitization induces the production of cytokines and chemokines in the skin, which stimulate the migration of Langerhans cells. The Slit2-mediated inhibition of the migration of Langerhans cells in the skin explant cultures could, therefore, be due to an effect on the production of cytokines and chemokines rather than a direct interaction of Slit2 with the Langerhans cells. To determine whether Slit2 can interact directly with DC, we analyzed the effects of Slit2 pretreatment on a Langerhans cell line (XS106) and a bone marrow-derived DC population, which contains >90% CD11c-positive DC (29, 30). After pretreatment, the cells were injected s.c. into mice, and the migration of the cells into the draining lymph nodes was assessed. In comparison with controls, pretreatment with Slit2 significantly reduced the migration of the bone marrow-derived DC and the XS106 cells into the draining lymph nodes ($p < 0.01$) (Fig. 3, *A* and *B*). Thus, Slit2 can interact directly with DC and inhibit their migration.

Pretreatment with Slit2 did not affect the proliferation of the XS106 cells (Fig. 3*C*). Additional experiments demonstrated that Slit2 treatment did not affect the ability of XS106 and bone marrow-derived DC to adhere on endothelial cells and fibronectin-coated plates (data not shown). These results implicate that Slit2 inhibits the motility of Langerhans cells and DC and does not have any toxic effect on the cells.

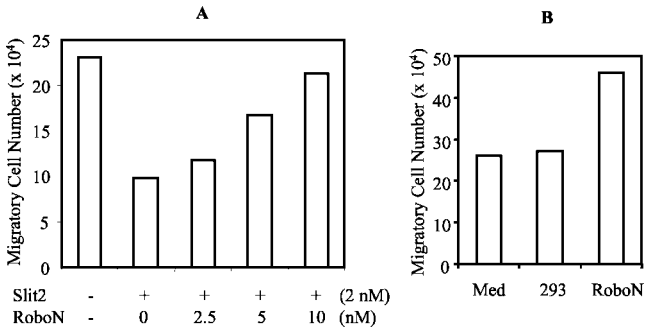

FIGURE 2. The inhibitory effect of Slit2 on hapten-induced Langerhans cell migration is mediated by the receptor, Robos. Skin explant cultures of DNFB-treated mouse ears were prepared as described in Fig. 1, with the addition of the soluble receptor RoboN in the culture medium. *A*, The neutralization effect of RoboN is dose dependent, and Langerhans cell migration in the presence of Slit2 can be completely rescued by RoboN. The experiment was repeated twice. *B*, RoboN (5 nM) neutralizes the activity of endogenous Slits and enhances the migration of Langerhans cells. Standard culture medium (Med) and HEK293-conditioned medium (293) served as controls. The experiment was repeated twice. Results represent the average of eight ear explants per group.

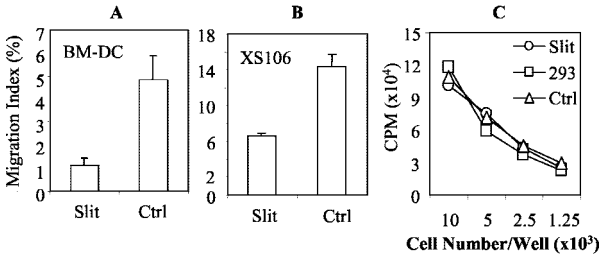

FIGURE 3. The inhibitory effect of Slit2 on DC is mediated via a direct interaction with the cells. Bone marrow-derived (BM) DC (*A*) or Langerhans cell line XS106 (*B*) were pretreated with Slit2 and then injected s.c. into mice. The migration index of Slit2-treated and untreated control (Ctrl) cells in the draining lymph nodes was evaluated 18–20 h after injection. The migration index of Slit2-pretreated XS106 and bone marrow-derived DC was significantly lower than that of the untreated counterparts ($p < 0.01$). Data represent the average of six mice per group. *C*, The treatment with Slit2 did not affect the proliferation of XS106 cells ($p > 0.05$). The experiments were repeated two to three times.

Slit2 inhibits the development of CHS responses

The development of CHS responses is dependent on hapten-carrying Langerhans cells that migrate from the hapten-treated skin into the draining lymph nodes where they present hapten to, and activate, specific T cells. Inhibition of Langerhans cell migration in the skin reduces the activation of hapten-specific T cells in the draining lymph nodes and attenuates CHS responses (15, 17, 36). To determine whether Slit2-mediated inhibition of the migration of Langerhans cells can modulate the development of CHS responses, purified Slit2, RoboN, or PBS was injected s.c. into the dorsal side of one ear, which was then painted with the hapten, oxazolone. The other ear of the mouse was then challenged with oxazolone 5 days later, and the CHS response in this ear was measured. Our preliminary experiments showed that oxazolone was a stronger hapten than DNFB and that one application of 10 μl on one ear could induce a significant level of CHS responses in mice (data not shown). We found that the CHS responses were attenuated significantly by application of Slit2 in comparison with the responses observed in control mice that were either treated with PBS alone or left untreated (Fig. 4; $p < 0.01$). Conversely, application of RoboN

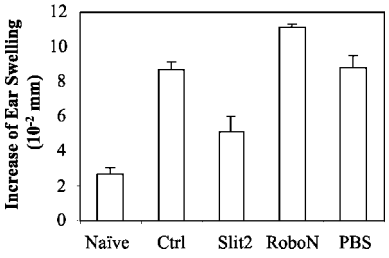

FIGURE 4. Slit2 inhibits contact hypersensitivity responses. Purified Slit2, RoboN, or PBS were injected into the dorsal site of one ear. Untreated mice served as control (Ctrl). The injected ear was then painted with the hapten, oxazolone. The other ear was challenged with the same hapten 5 days later, and the ear swelling response was measured. Controls include naive mice that were not sensitized to hapten but were challenged. Slit2 significantly reduced the inflammatory response ($p < 0.01$), whereas application of RoboN heightened the response considerably ($p < 0.05$). Data represent the average of four mice in each group. The experiment was repeated four times.

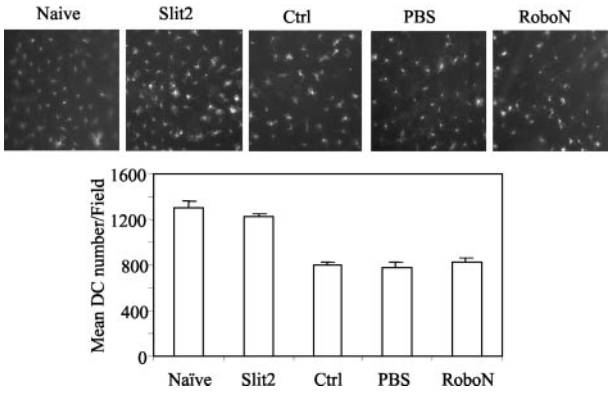

FIGURE 5. Slit2 inhibits the hapten-induced migration of Langerhans cells in vivo. Purified Slit2, RoboN, or PBS was injected into the dorsal site of ears. Mice without injection in the ear served as controls (Ctrl). The injected ear was then painted with oxazolone. The skin samples were harvested 18–20 h later, and Langerhans cells in the epidermis were stained with PE-labeled anti-Ia/IE Ab. Naive skin served as a control. *A*, Representative photomicrographs of the epidermis of naive, untreated control or Slit2-, PBS-, and RoboN-treated samples. *B*, The number of Langerhans cells in the epidermis of skin samples was counted. A significant difference in the number of Langerhans cells in Slit2-treated skin samples and the numbers in PBS-, control, or RoboN-treated skin samples was observed ($p < 0.05$). The Langerhans cell numbers represent the average of six ears per group. The experiment was repeated three times.

exacerbated the CHS response ($p < 0.05$). These results demonstrate that Slit2 can suppress the development of CHS responses in mice and that neutralization of Slit activity enhances the response.

To determine whether the Slit2-mediated reduction of the CHS response is associated with inhibition of the migration of Langerhans cells, we evaluated the numbers of Langerhans cells in the treated skin using anti-MHC class II Abs. At 18–20 h after hapten painting, the number of Langerhans cells in the hapten-treated epidermal sheets was significantly lower than the number in naive skin ($p < 0.01$), confirming that hapten sensitization drives the migration of the Langerhans cells out of the skin (Fig. 5). The number of cells in the Slit2-treated ear skin was comparable with the number in naive skin, indicating that hapten-induced migration of Langerhans cells out of the skin is inhibited by Slit2. Somewhat surprisingly, treatment with RoboN did not significantly affect the number of Langerhans cells in the hapten-treated epidermis ($p > 0.05$), even though this treatment had been shown to exacerbate hapten-induced CHS responses.

Expression of Slits in the skin

The expression of Slits in the skin of adult mice has not been evaluated to date, and stringent analysis is not yet possible due to the lack of Slit-specific Abs. A preliminary evaluation was possible, however, through the detection of Slit mRNA using RT-PCR with specific primers for mouse Slit1, -2, and -3. In concordance with the published reports, all three types of Slit mRNA were detected in the mouse brain tissue that was used as a positive control (Fig. 6*A*). We were able to show that Slit2 mRNA is expressed constitutively in adult mouse skin. Neither Slit1 mRNA nor Slit3 mRNA were detected using this assay.

The effect of cutaneous inflammation on the expression of Slit mRNA was determined at 4, 24, and 48 h after epicutaneous sensitization of the mice with DNFB. The expression of Slit2 increased after hapten sensitization, although Slit1 and Slit3 remained undetectable. Quantitative analysis using real time RT-PCR indicated that the expression of Slit2 mRNA was 2- to 3-fold higher after hapten sensitization (Fig. 6*B*). This rapid up-regulation of Slit2 afater hapten sensitization suggests that Slit2-mediated effects in the skin can occur during the early phases of the inflammatory reaction.

To determine what cells in the skin produced Slits, the expression of Slit2 in the naive and hapten-treated skin was detected by in situ hybridization. The naive skin expressed a low to undetectable level of Slit2. Sensitization with hapten oxazolone (24 h) induced a high level of Slit2 expression in the epidermis. The controls with sense cRNA did not reveal any staining. Slit2 expression

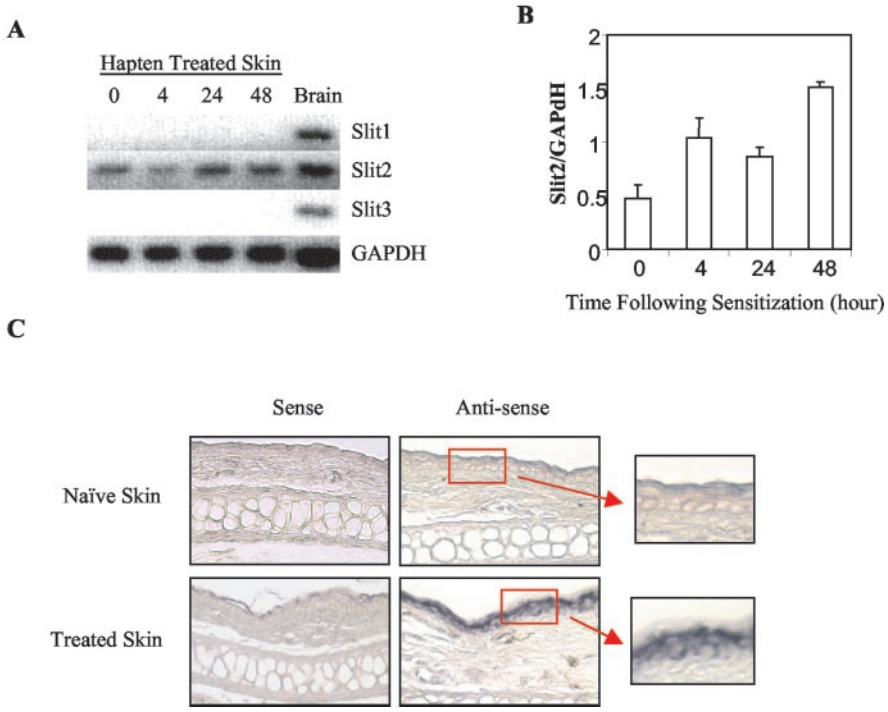

FIGURE 6. Expression of Slits in the skin. *A*, The reported expression of Slit1, -2, and -3 mRNA in the mouse brain was confirmed. Slit2 mRNA was found to be expressed constitutively in the naive skin and up-regulated at 4, 24, and 48 h after hapten sensitization. Slit1 and Slit3 were not detectable in the naive or hapten-treated skin using this assay. Representative figures are shown from four experiments. *B*, Slit2 mRNA in the skin was quantitatively determined by real time RT-PCR after sensitization with DFNB. *C*, In situ hybridization indicated that Slit2 was expressed in the epidermis of sensitized mice but undetectable in naive animals. The specific signal of Slit2 was not detected in the dermis. Sense cRNA served as controls.

in the dermis was not detectable in the naive as well as hapten-treated animals (Fig. 6*C*), suggesting that keratinocytes may be the major source of Slit2 in the skin. RT-PCR analysis exhibited a significant level of expression of Slit2 in the naive skin. The discrepancy may reflect a difference in the sensitivity between two assays.

XS106, bone marrow-derived DC, migratory cells from skin explant cultures, and leukocytes purified from the spleen did not express any form of Slits (data not shown).

Expression of Robo1 by DC

The receptors for Slits are Robo molecules, Robo1, -2, and -3 (37, 38). Robo1, which is expressed on leukocytes and in lymphoid organs, has been proved to mediate the inhibitory effects of Slit2 on leukocyte chemotaxis (20). It has yet to be determined whether DC express Robos.

To examine Robo expression by DC, we have generated a panel of chicken Abs against synthetic Robo1 peptides and applied them to stained cells by immunocytochemical technique. One Ab raised to peptide 106–119 of Robo1 (identical for mouse and rat Robo1) stained RoboN (the extracellular domain of rat Robo1)-transfected 293 cells but did not react with the parental cells. Furthermore, the reactivity of the Ab to RoboN-transfected 293 cells was diminished by preabsorption with soluble recombinant RoboN (Fig. 7*A*). These data demonstrate that this Ab specifically stains Robo1 proteins.

To detect whether mouse DC express Robo1, XS106 and bone marrow-derived DC were stained with the Ab. Both cells were stained, which could be blocked by preincubation of the Ab with the recombinant RoboN (Fig. 7*B*). The staining on migratory cells from skin explant cultures exhibited that most of the cells were Robo1 positive. Further analysis with double staining with anti-Ia/IE Ab demonstrated that most MHC class II-positive cells expressed Robo1. However, some MHC class II-negative cells also expressed Robo1. There were Robo1-positive cells that expressed CD11b, suggesting that monocytes and granulocytes may express Robo1 as well (Fig. 7*C*). Immunohistochemical staining with the Ab did not reveal a detectable level of Robo1 expression on skin cross-sections as well as epidermal sheets of naive mice (data not shown), suggesting that Robo1 expression by DC may be induced by inflammatory stimuli.

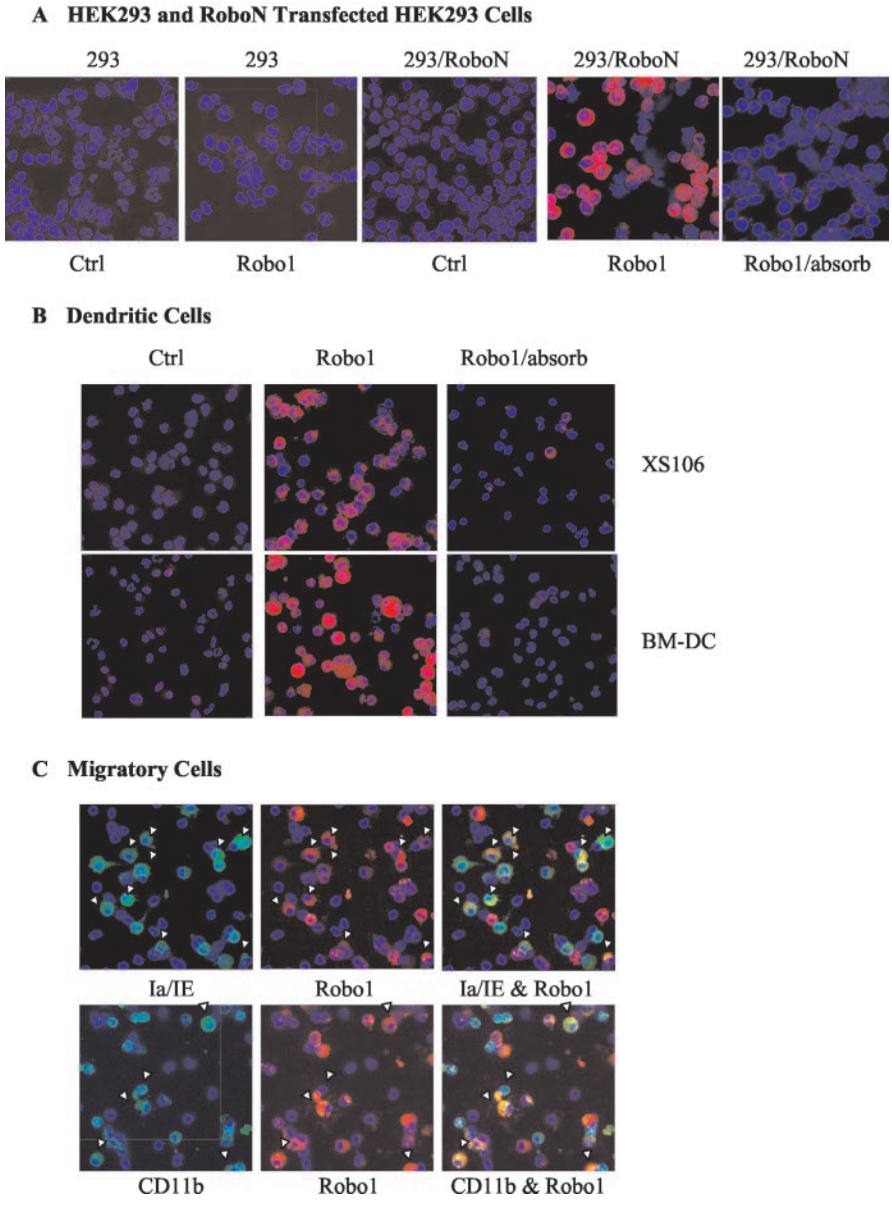

FIGURE 7. Expression of Robo1 by DC. Cells were stained with chicken anti-Robo1 Ab followed by Cy-3-labeled anti-chicken Ab (red). DAPI was used to stain nucleus (blue). *A*, The Ab stained RoboN-transfected but not wild-type HEK293 cells. The staining of RoboN-transfected cells with the Ab was diminished by preabsorption with soluble RoboN. *B*, Anti-Robo1 Ab stained XS106 and bone marrow-derived (BM) DC. Preabsorption of the Ab with RoboN diminished the staining. *C*, The majority of migratory cells from skin explant cultures were stained by the anti-Robo1 Ab. Double staining with anti-Ia/IE Ab (green) showed that most of MHC class II-positive migratory cells expressed Robo1 (arrows). Some Robo1-positive cells were stained by CD11b Ab (green, arrows).

Taken together, these results indicate that DC, including migratory Langerhans cells, express Robo1 and are capable of responding to Slits2 that is not only expressed in the skin but also up-regulated during allergen-mediated inflammatory reactions.

Discussion

We have demonstrated that Slit2, which was originally described as a neuronal repellent factor, is capable of inhibiting the migration of Langerhans cells out of the skin and that the Slit2-mediated inhibition of Langerhans cell migration suppresses the development of CHS responses. The data provide the first evidence demonstrating Slit2 as an anti-inflammatory factor in the regulation of immune responses.

Migration of Langerhans cells from the skin to the regional lymph nodes is the initial event in the development of hapten-induced contact hypersensitivity. It is well established that, following hapten sensitization, inflammatory cytokines and chemokines are induced in the skin, which activate Langerhans cells and stimulate their migration out of the skin (14, 36, 39). Previous studies from the laboratories of other investigators as well as our own have demonstrated that the number of Langerhans cells in the epidermis is reduced after exposure to hapten (30, 40). At the same time, there is an influx of Ag-carrying DC into the draining lymph nodes. The regulation of Langerhans cell migration in the skin affects the magnitude of CHS responses (15, 19, 41). The results of the current study demonstrate clearly that the inhibitory factor, Slit2, can both inhibit the hapten-induced migration of Langerhans cells and suppress the CHS response. It is unknown at this time whether Slit2 inhibits the CHS response solely through its effects on the migration of the Langerhans cells, as it is possible that it affects the maturation of the Langerhans cells or their ability to stimulate T cells.

Down-regulation of the hapten-induced migration of DC is known to occur in vivo. Within 24 h of hapten sensitization, the number of Langerhans cells in the epidermis is largely recovered, whereas the number of hapten-carrying DC in the regional lymph nodes declines after 24 h (30, 42). A simple and reasonable explanation for this down-regulation is that the production of the stimulatory factors that are involved in the activation of the DC is reduced on effective elimination of the haptens. It is possible, however, that inhibitory factors, such as Slit2, are produced and are capable of rapidly and effectively down-modulating the migration. We found that the expression of Slit2 in the skin was up-regulated significantly as early as 4 h after hapten sensitization, indicating that the induction of this inhibitory factor occurs during the early stages of the response and at the same time as the induction of proinflammatory cytokines and chemokines (43). We found that neutralization of Slit2 with a soluble form of its receptor enhanced the migration of the hapten-carrying Langerhans cells and exacerbated the CHS response. Taken together with the finding that Slit2 is expressed constitutively in the skin and that its levels are elevated during the hapten-induced inflammatory response, these data imply the existence of a previously undescribed anti-inflammatory mechanism that limits Ag presentation of DC to T cells.

The number of Langerhans cells in the epidermis reflects the balance of cell migration into and out of the site. During inflammation induced by hapten sensitization, Langerhans cells migrate out of the skin and move into the draining lymph nodes. At the same time, fresh Langerhans cells are recruited into the inflammatory skin, most likely from the blood (44). In this study, we have established that Slit2 inhibits the migration of Langerhans cells out of the skin, resulting in the retention of the cells in the skin. We expected that neutralization of Slits in the skin by treatment with a soluble form of their receptor, RoboN, would enhance the migration of Langerhans cells out of the skin and therefore that the number of Langerhans cells in the epidermis would be lower than that in the untreated controls. Although an enhanced CHS response in the RoboN-treated mice provided evidence of the migration of hapten-carrying Langerhans cells into the regional lymph nodes, the anticipated reduction in the number of Langerhans cells in the RoboN-treated skin was not observed. This apparent discrepancy may simply reflect enhanced recruitment of Langerhans cells on the neutralization of Slits. The issue requires further investigation.

The constitutive expression of Slit2 and rapid up-regulation after allergen stimulation in the skin may play a role in the retention of Langerhans cells under steady state conditions. As the skin encounters enormous amounts of environmental Ags, most of which are innocuous, the ability of the body to ignore specific Ags is critical. That is, inflammatory reactions are not induced by most of the Ags that are encountered. Under steady state conditions, Langerhans cells do migrate from the skin to the draining lymph nodes, but only at a low rate (45, 46). It is possible that the constitutive expression of Slit2 in the skin contributes to these low rates of migration and that Slit2 may be an important mechanism in preventing the inappropriate development of cutaneous inflammation.

Robo1 is expressed not only by MHC class II-positive Langerhans cells and DC but also by MHC class II-negative cells. Some Robo1-positive cells express CD11b, a marker for monocytes and granulocytes, suggesting that the migration of other leukocytes during inflammation may be regulated by Slits. Characterization of Robo1-positive leukocytes in inflammatory tissues and examination of Slits in the regulation of inflammatory reactions at the elicitation phase are in progress.

The directional movement and motility of DC is regulated by chemokines (9, 47). Slit2 has been demonstrated to inhibit chemokine-mediated chemotaxis of leukocytes (20). This mechanism may play a role in the regulation of DC migration as well. It remains to be identified whether Robos cross-talk with chemokine receptors in the immune system and whether activation of Robos affects the function of chemokine receptors that are related to the regulation of directional migration of DC. A recent study has demonstrated that the interaction of Slit2 with the Robo1 receptor inhibits the activity of Cdc42 (38), a member of the Rho family of small GTPases that play important roles in adhesion and migration of leukocytes (48). Currently, the role of Rho GTPases in the regulation of DC adhesion and migration is largely undefined, but a recent report has indicated that the directional movement of DC may be initiated by Cdc42-mediated polarization and extension of filopodia (49). Further studies are required to identify the molecular mechanisms underlying Slit2-mediated inhibition of DC migration.

In summary, Slit2 is expressed in mouse skin, and its expression is up-regulated on hapten sensitization. DC and migratory Langerhans cells express Robo1, a ligand for Slits. Slit2 inhibits the migration of Langerhans cells and suppresses the development of CHS responses after hapten sensitization. This study sheds light on a novel mechanism that regulates the immune response during the early phase of the response by limiting the migration of APCs to lymphoid tissues. These findings suggest that application of Slit2 has potential value as an anti-inflammatory strategy in the prevention and treatment of immune response-mediated diseases.

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