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*J Immunol* 2001; 166:7571-7578; doi: 10.4049/jimmunol.166.12.7571
http://www.jimmunol.org/content/166/12/7571
Eotaxin (CCL11) Induces In Vivo Angiogenic Responses by Human CCR3+ Endothelial Cells

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Chemokines are attractants and regulators of cell activation. Several CXC family chemokine members induce angiogenesis and promote tumor growth. In contrast, the only CC chemokine, reported to play a direct role in angiogenesis is monocyte-chemotactic protein-1. Here we report that another CC chemokine, eotaxin (also known as CCL11), also induced chemotaxis of human microvascular endothelial cells. CCL11-induced chemotactic responses were comparable with those induced by monocyte-chemotactic protein-1 (CCL2), but lower than those induced by stroma-derived factor-1α (CXCL12) and IL-8 (CXCL8). The chemotactic activity was consistent with the expression of CCR3, the receptor for CCL11, on human microvascular endothelial cells and was inhibited by mAbs to either human CCL11 or human CCR3. CCL11 also induced the formation of blood vessels in vivo as assessed by the chick chorioallantoic membrane and Matrigel plug assays. The angiogenic response induced by CCL11 was about one-half of that induced by basic fibroblast factor, and it was accompanied by an inflammatory infiltrate, which consisted predominantly of eosinophils. Because the rat aortic sprouting assay, which is not infiltrated by eosinophils, yielded a positive response to CCL11, this angiogenic response appears to be direct and is not mediated by eosinophil products. This suggests that CCL11 may contribute to angiogenesis in conditions characterized by increased CCL11 production and eosinophil infiltration such as Hodgkin’s lymphoma, nasal polyposis, endometriosis, and allergic diathesis.


Received for publication January 8, 2001. Accepted for publication April 12, 2001.

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1 This project was funded in part with Federal funds from the National Cancer Institute, National Institutes of Health, under Contract NO1-CO-56000. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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3 Abbreviations used in this paper: MCPs, monocyte-chemotactic proteins; HMECs, human microvascular endothelial cells; EGF, epidermal growth factor; SDF-1α, stroma-derived factor-1; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; BM, binding medium; CAM, chorioallantoic membrane.

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0022-1767/01/$02.00
question whether eosinophil products are responsible for angiogenesis or are due to a direct effect of CCL11 on endothelial cells. On the basis of our data, CCL11 has the potential to be a direct mediator of angiogenesis, as measured by its ability to induce in vitro endothelial cell migration and in vivo angiogenesis in the Matrigel plug and in CAM assays. Furthermore, CCL11 induced endothelial cell sprouting from aortic rings in the absence of an eosinophil infiltrate. These results demonstrate that CCL11 directly mediates angiogenesis.

Materials and Methods

Chemokines and Abs

Recombinant human CCL11, recombinant human vascular endothelial growth factor (VEGF), and recombinant human basic fibroblast growth factor (bFGF), recombinant human SDF-1α (CXCL12), recombinant human MCP-1 (CCL2) and recombinant human IL-8 (CXCL8) were purchased from PeproTech (Rochester, NY). Endothelial cell growth supplement was purchased from Sigma (St. Louis, MO). Mouse anti-human CCL11 and monoclonal rat anti-human CCR3 was purchased from R&D Systems (Minneapolis, MN), and mouse IgG and rat IgG (Coulter, Miami, FL) were used as the negative controls.

Cell culture

HMECs were obtained from CSC (Kirkland, WA). Endothelial cell cultures were tested for their expression of CD31 and von Willebrand factor by flow cytometry, and preparations containing <2% contaminating cell types were selected for further studies. Endothelial cells were cultured on collagen type I-coated plastic wells (Biocoat; BD Biosciences, Mountain View, CA), in endothelial growth medium (Clonetics, Walkersville, MD) containing 5% FCS, VEGF (10 ng/ml), bFGF (10 ng/ml), glutamine (2 mM), and gentamicin (100 U/ml). All experiments were performed using subcultures between the second and seventh passages. Human eosinophils were isolated from Leukopaks by Percoll gradient centrifugation followed by MACs CD16 negative selection (Miltenyi Biotec, Auburn, CA).

Flow cytometric analysis

Indirect immunofluorescence was performed on HMECs by exposing cells to saturating amounts of rat Abs to human CCR3. Fluorescein-conjugated F(ab)′2, fragments of goat anti-rat (Sigma) diluted 1/100 were used as the secondary Ab. After staining, cells were analyzed using a FACScan flow cytometer (BD Biosciences).

Endothelial cell migration assay

HMEC and human eosinophil chemotaxis was performed using microBoyden chambers. Briefly, polycarbonate filters of 5 μm pore size (Nuclepore; NeuroProbe, Cabin John, MD) were coated with fibronectin or collagen I (10 μg/ml; Sigma) overnight at 4°C. Binding medium (BM) containing 1% BSA in RPMI 1640 with or without various amounts of chemokine including CCL11, SDF-1α (CXCL12), MCP-1 (CCL2), and IL-8 (CXCL8) was placed in the lower compartment of the chamber, and 50 μl HMECs resuspended at a concentration of 0.5 × 10⁵ cells/ml in BM were then added to the upper compartment. The chambers were incubated for 2 h for eosinophil chemotaxis and 3–4 h for HMEC chemotaxis at 37°C. After the filters were removed, the upper surface was scraped, fixed with methanol, and stained with Leukostat (Fisher Scientific, Pittsburgh, PA). Membranes were analyzed using the BIOQUANT program (R & M Biometrics, Nashville, TN), and the results were expressed as the mean number of migrated cells/ten fields at ×10 magnification. For inhibitory assays, human CCL11 Ab was added together with CCL11 in the lower compartment of the chamber. Anti-human CCR3 Ab was added to HMECs or human eosinophils 10 min before chemotaxis was performed. Each sample was tested in triplicate. Chemotaxis and inhibition of chemotaxis experiments were performed five times.

Endothelial cell proliferation assay

HMECs were resuspended at 1 × 10⁵ cells/ml of proliferation medium (RPMI, 0.5% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). 50 μl of the cell suspension/well was placed in a gelatin-coated 96-well plate. Cells were stimulated with different concentrations of CCL11, bFGF (10 ng/ml), VEGF (300 ng/ml). Plates were incubated at 37°C in 5% CO₂ for 48 h. To determine cell proliferation, cells were incubated with [³H]thymidine (0.5 to 1 μCi/well) 18 h before uptake determination. After the incubation, plates were kept at −70°C overnight; finally, the plates were thawed at room temperature and harvested, and [³H]thymidine incorporation was determined using a beta counter.

RNase protection assay

HMECs were grown in EBM medium containing 5 ng/ml recombinant human epidermal growth factor (EGF). RNA was isolated by the TRIzol method and directed (Life Technologies, Gaithersburg, MD) and thereafter used for analysis of mRNA expression by the Riboquant RNase Protection Assay System (human CR6 probe set; Pharmingen, San Diego, CA) according to the manufacturer’s instructions. Briefly, ³²P-labeled antisense RNA probes were synthesized from the human chemokine receptor 5 (CC chemokine receptor) template by T₇ RNA polymerase. The probe (1.5 × 10⁵ cpms) was hybridized in solution overnight in excess to target RNA (10 μg total RNA/treatment) in a total reaction volume of 10 μl. The free probe and other single-stranded RNA were digested with RNases A and T₁, per instructions provided by the manufacturer. The remaining RNase-protected probes were precipitated, dissolved in 3 μl sample buffer (Pharmingen), and resolved on denaturing polyacrylamide gels followed by autoradiography for 1–7 days at −70°C.

Rat aortic ring assay

The thoracic aorta was obtained from 100- to 150-g male Sprague Dawley rats (Taconic Farms, Germantown, NY). Excess peripheral tissue was removed, transverse sections (1–2 mm) were made, and the resulting aortic rings were then washed in medium 199 (Life Technologies). The rings were then embedded in Matrigel (BD Biosciences) in eight-well chamber slides (Nalge Nunc International, Milwaukee, WI) so that the lumen was parallel to the base of the slide. After the Matrigel gelled, serum-free medium (endothelial basal medium supplemented with antibiotics) with or without different concentrations of CCL11 (0.1–100 nM) was added to each well, and the slides were incubated at 37°C, with 5% CO₂, for 3 days. (n = 6 per dose). Endothelial cell growth supplement or bFGF were used as the positive controls at concentrations of 10 μg/ml or 60 nM, respectively. For inhibition experiments, Abs to CCL11 or control mouse IgG (10 μg/ml) were added simultaneously with CCL11. After the incubation period, the rings were fixed, stained, and photographed. The ring assay was repeated four times.

Chick chorioallantoic membrane (CAM) assay

OVA (4 ml) was removed from 3-day-old embryonated eggs (Truslow Farms, Charlestown, MD). Thereafter, windows were opened for each egg, coated with tape to prevent drying, and eggs were incubated at 37°C. On day 10, 5 μl distilled water containing different amounts of CCL11 were applied in the center of quartered 13-mm-diameter plastic coverslips (Thermanox; Nalge Nunc International) and dried for 10 min at 37°C. Each coverslip was placed on the CAM of the chick, and the eggs were incubated at 37°C for 3 days. The assay was scored by a blinded observer and photographed on the 13th embryonic day. Thereafter, the CAMs were cut, fixed with 4% paraformaldehyde, and stained with either hematoxylin and eosin or Giemsa to evaluate the inflammatory infiltrate. bFGF (10 ng) and water were used as positive and negative controls, respectively. Twenty eggs were used in total for each data point. A positive score for angiogenesis was made when vessels appeared to radiate from the spot in the coverslip to which the stimulant was applied. The scores are reported as a percentage of positive CAMs at each dilution. The experiment was repeated twice.

In vivo Matrigel plug angiogenesis assay

Matrigel (9 mg/ml; 0.3 ml/mouse) alone or mixed with different concentrations of CCL11 was injected s.c. into the flank of C57BL/6 mice. On day 7, mice were sacrificed, and plugs were removed, fixed in 3.7% formaldehyde, paraffin embedded, and hematoxylin-eosin- or Giemsa-stained slides were photographed. The experiment was repeated twice with eight mice per group in each experiment. For quantification of angiogenesis, the procedure described by Hoffman et al. (16) was used, with modifications. Briefly, 1% of FITC-conjugated dextran (100 mg/kg; Sigma) in PBS (0.2 ml/mouse) was injected i.v. in the tail vein of mice, 20 min before the extraction of the Matrigel. Matrigel sections were weighed and boiled in 5 N HCl, and the fluorescence was read in a fluorometer by excitation at 485 nm and emission at 530 nm. Some Matrigel sections were fixed in buffered formaldehyde and photographed using a fluorescence microscope.

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Results

HMECs migrate toward CCL11 and express CCR3

We evaluated the ability of human CCL11 to induce in vitro chemotaxis of HMECs. As shown in Fig. 1, we observed a dose-dependent chemotactic response of HMECs toward CCL11. The maximal chemotactic response for each of the HMECs and human eosinophils was observed at 10 nM CCL11 (Fig. 1A). The chemotactic response of human eosinophils, which were used as the positive control, was at least 2-fold higher than the response of HMECs (Fig. 1A). A checkerboard analysis indicated that the migratory effect of CCL11 on HMECs is chemotactic rather than chemokinetic (Table I). The specificity of this chemotactic response was determined using a blocking mAb to human CCL11. This assay indicated that the chemotactic response of HMECs toward CCL11 was blocked by CCL11 mAb at concentrations of 20 µg/ml (Fig. 1B). Both human and murine CCL11 elicited similar chemotactic responses by HMECs (Fig. 2C) and heat inactivation completely abolished the activity of either human or murine CCL11 (Fig. 2C). Because several chemokines have been shown to induce responses by endothelial cells, we compared the chemotactic response induced by CCL11 with other chemokines including SDF-1α, CXCL12), MCP-1 (CCL2), and IL-8 (CXCL8). As shown in Fig. 1D, CCL11 responses were comparable with CCL2 responses, but were 3- and 1.5-fold lower than those induced by CXCL12 and CXCL8, respectively. Because several angiogenic factors also induce endothelial cell proliferation, we tested whether CCL11 has a proliferative effect. In contrast to VEGF and bFGF, CCL11 did not induce in vitro proliferation of HMECs (Table II).

Because the responsiveness of HMECs to CCL11 suggests the expression of CCL11 receptor on endothelial cells, we used the RNase protection assay which allows for the detection of multiple chemokine receptor mRNAs in a single RNA preparation. As shown in Fig. 2A, HMECs expressed low levels of CCR3 mRNA. Additionally, expression of CCR1, CCR2, and CCR4 mRNA was observed. The expression of CCR3 mRNA was not up-regulated by VEGF or bFGF stimulation (data not shown). We further analyzed the expression level of CCR3 protein, the receptor for CCL11, by HMECs. Using immunofluorescence, we detected CCR3 on the cell surface of HMECs (Fig. 2B). The mean fluorescence intensity was 48 (±16) for HMECs vs 158 (±32) for human eosinophils. The expression of CCR3 on HMECs was at least 4-fold lower than that found on human eosinophils (Fig. 2C). Blocking experiments indicated that the CCL11-induced chemotactic responses by HMECs were partially inhibited (75%) by anti-human CCR3 (Fig. 2D), whereas the chemotactic responses by eosinophils were completely abolished by anti-CCR3, suggesting the existence of other receptors(s) for CCL11 on HMECs.

| Table 1. Checkerboard analysis of eotaxin (CCL11)-induced HMEC chemotaxis* |
|------------------|------------------|------------------|------------------|
| hCCL11 in Lower Well (nM) | hCCL11 in Upper Well (nM) |
| 0 | 30 ± 8 |
| 0.1 | 48 ± 17 |
| 1 | 110 ± 5* |
| 10 | 127 ± 11* |

* Human CCL11 (hCCL11) at specified concentrations were added to the lower wells of the chemotaxis chamber. HMECs (0.5 × 10⁶ cells/ml) in the absence or presence of specified concentrations of human CCL11 were added to the upper wells of the chemotaxis chamber. The average (mean ± SEM) of migrated HMECs of triplicate wells is shown as the number of cells per high power field. *p < 0.01 and **p < 0.05 as compared with spontaneous HMECs migration (in bold type).

FIGURE 1. HMECs migrate toward eotaxin (CCL11). A. The migration of HMECs and eosinophils toward different concentrations of CCL11 was quantitated as the number of cells per ×10 field as described in Materials and Methods. *p < 0.001; **p < 0.05. B, Inhibition of the chemotactic responses of HMECs toward CCL11 by anti-eotaxin (20 µg/ml) was tested at the dose of CCL11 that induced maximal chemotactic responses (10 nM) and compared with that induced by BM alone. *p < 0.001. C. The chemotactic responses induced by human (h-) and murine (m-) eotaxin (CCL11) were compared. Heat (△) inactivation of human and murine eotaxin (CCL11) completely abolished CCL11 chemotactic activity. D. CCL11 chemotactic responses are comparable with those of MCP-1 (CCL2). Comparative analysis was performed as described in Materials and Methods. The mean and SEM of two experiments are shown. *p < 0.01; **p < 0.05.
Human CCL11 induces angiogenesis in vivo

To evaluate whether CCL11 could exhibit angiogenic activities in vivo, we tested different doses of CCL11 ranging from 1 to 100 ng, using the CAM assay. As shown in Fig. 3, CCL11 induced angiogenesis in the CAM assay. The maximal angiogenic responses were obtained at 100 ng (78%) (Fig. 3, C and D) and 10 ng (63%) of CCL11 (Fig. 3, B and D). No significant angiogenic response was obtained at a dose of 1 ng CCL11 (Fig. 3D). The negative control showed <20% positivity (Fig. 3, A and D). As expected, an inflammatory infiltrate composed of heterophils and eosinophils was observed in association with the angiogenic effect induced by CCL11 as shown by the histological sections of the CAMs (Fig. 3F). An absence of this inflammatory response was observed in the negative control (Fig. 3E). These data demonstrate that CCL11 has angiogenic effects in vivo and suggest the existence of a chick receptor functionally homologous to the human CCR3.

We also evaluated the effect of CCL11 using the in vivo Matrigel plug assay. Mice were injected with Matrigel alone or with Matrigel containing CCL11, s.c. in the flank region. Histological sections of the Matrigel plugs indicated a significant angiogenic effect induced by CCL11 when used at a concentration between 10 and 100 nM (Fig. 3B, C, F, G, and I), in contrast to Matrigel alone (Fig. 4, A, E, and I). Furthermore, as with the CAM assay, an inflammatory reaction was also observed in the Matrigel plugs containing CCL11, which consisted predominantly of eosinophils (Fig. 4D). Quantitative studies using human (Fig. 4, F and J) and murine (Fig. 4, G and I) CCL11 indicated similar angiogenic responses, that were about one-half as potent as the angiogenic response induced by human bFGF in vivo (Fig. 4, H and I).

CCL11 induced rat aortic endothelial cell sprouting

We used the ex vivo rat aortic ring-sprouting assay, which allows the detection of angiogenesis in the absence of an inflammatory response to rule out the possibility that the angiogenic responses mediated by CCL11 were a result of eosinophilic products. Transverse sections of rat aorta tissue embedded in Matrigel were cultured with different concentrations of CCL11 as described in Materials and Methods and thereafter examined for the degree of sprouting vessels. As shown in Fig. 5, CCL11 stimulated numerous capillary sprouts at 1 nM (Fig. 5E) and 10 nM (Fig. 5, B and E). Thus, CCL11 can induce endothelial cell sprouting at nanomolar concentrations from rat aortic rings in the absence of eosinophils, favoring a direct effect in promoting angiogenesis. Additionally, the angiogenic response induced by CCL11 was markedly inhibited by a mAb to CCL11 (Fig. 5, C and E).

Discussion

Eotaxin (CCL11) is abundantly expressed in the mucous membranes during allergic inflammation, in which angiogenesis also occurs. In this process, it is thought that CCL11-mediated angiogenesis is dependent on eosinophil and mast cell products including TGF-α and -β (17) and chymase, respectively (18, 19), which are also potent angiogenic factors. Additionally, CCL11 expression during endometriosis correlated with eosinophil degranulation and wound healing (20). CCR3, the major receptor for CCL11, has been reported to be expressed by CNS endothelial cells (12, 13).

On the basis of the above evidence, we tested the hypothesis that CCL11 also directly mediates angiogenic responses. Using in vitro and in vivo assays, we demonstrated that CCL11 induces angiogenic responses by human, mouse, rat, and chick endothelial cells. In vitro, CCL11 induced human endothelial cell migration in a dose-responsive manner that was correlated with the expression of...
CCR3 at the RNA and protein levels. In contrast to CXCR4, the expression of CCR3 by human endothelial cells was not up-regulated by classical angiogenic factors such as VEGF or bFGF (data not shown). The in vivo angiogenic effect of CCL11 was associated with an inflammatory infiltrate comprised mostly of eosinophils. However, because angiogenesis induced by eosinophil products has been proposed, we used the rat aortic ring assay to evaluate angiogenic effects in the absence of an inflammatory

**FIGURE 3.** Eotaxin (CCL11) promotes angiogenesis in the chick CAM assay. Plastic coverslips containing 10 (B) or 100 (C) ng of CCL11 were placed on the chorioallantoic membrane of 10-day-old embryos. Distilled water, which was used as a solvent, served as the negative control (A). The percentage of positive CAMs for each CCL11 concentration was scored. bFGF (10 ng) was used as a positive control (D). E and F, Eosinophils and heterophil infiltrates observed by Giemsa staining of CAM histological sections, control (E), and CCL11 at 100 ng (F). Twenty embryonated eggs were used for each data point, and the experiment was repeated twice. A representative experiment is shown.
FIGURE 4. Eotaxin (CCL11) induced angiogenesis in Matrigel plugs. Histological sections of Matrigel plugs after 7 days with or without CCL11 are shown. A, Matrigel plug section with PBS, which was used as the solvent. B, Matrigel plug containing 50 nM CCL11 (×10 magnification). C, High magnification of B (×40). S, stroma surrounding the plugs; M, Matrigel. Arrows, endothelial cells forming vessels in the Matrigel plug section containing CCL11. D, Giemsa staining of the Matrigel plug sections. As indicated by the arrows, eosinophil infiltration was associated with the angiogenic response induced by CCL11. Photographs were taken at ×400 magnification. E–I, Quantitative analysis of the angiogenic response induce by CCL11. FITC-dextran was used to assess the level of vascularity in Matrigel plugs as described in Materials and Methods. E, Matrigel plus vehicle (PBS). F, Human bFGF (60 nM). G, Murine CCL11 (10 nM). H, Human CCL11 (10 nM). Photographs were taken at ×20 magnification using a fluorescence microscope. I, Fluorometric quantification of the dextran fluorescence intensity (F.I.) obtained per 100 mg Matrigel implants. Matrigel plus vehicle (brown bars) F: human bFGF (green bars). G, Murine CCL11 (purple bars). H, Human CCL11 (blue bars). The mean and SEM of two experiments are shown. *, p < 0.001; **, p < 0.05.
CCL11 at 10 nM. Oral squamous cell carcinoma (30) and gastric carcinoma with squamous cell differentiation (31) constitute additional examples of eosinophil infiltration that correlate with an unfavorable prognosis. In contrast, infiltrating eosinophils in cervix (32), lung (33), colon carcinomas (34, 35), and murine mammary adenocarcinoma (36) have been considered as good prognostic indicators. Although the underlying basis of these divergent responses is unclear, perhaps it is based on heterogeneity in the cytokine repertoire of different tumor types. The contribution of CCL11 toward tumor angiogenesis should be determined to address this issue by testing the effect of inhibitors of CCL11 on tumor growth and other pathological conditions, such as allergic nasal polyposis, and allergic inflammatory states.

Although multiple chemokine receptors have been shown to be expressed by endothelial cells including CXCR-1, -2, -3 (37), CXCR4 (4, 38, 39), CCR2, CCR3 (12, 13), CCR8 (40), CCR4 and CCR5 (13), only CXCR4 plays a requisite role in angiogenesis as shown by the CXCR4 knockout (5). In contrast, disruption of the CCL11 gene apparently did not affect angiogenesis (41–43), presumably due to the redundancy of the chemokine network. The functional activities of CCL11 can also be compensated by eotaxin-2 (CCL24), eotaxin-3 (CCL26), MCP-4, or RANTES, which also interact with CCR3. Despite the plethora of identified angiogenic ligands, ligands for CCR3 may make potentially some unique contributions. The expression of multiple chemokine receptors by endothelial cells might reflect a steady state of the endothelium in an environment in which the balance or imbalance of inducers and inhibitors drives vasculature to develop or regress. For example, in situations like wound healing, in which “angiogenic” chemokines such as IL-8 and MCP-1 are generated, within the first hours, and “angiostatic” IP-10 and MIG are generated after 3 days of the healing processes (44); participation of different chemokine receptors with different specificities is apparently required to bring about normal wound healing (44). Many proinflammatory chemokines including CCL11 might not only promote inflammation but also support the necessary vascularity at inflammatory sites.

Acknowledgments

We thank Dr. Ken Wasserman for critically reviewing the manuscript and helpful suggestions. We also thank Nancy Dunlop, Douglas Halverson, and Keith Rogers for technical assistance.

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The role of CCL11 in the progression of cancer is controversial. In certain cases, such as Hodgkin’s tumors, the production of CCL11 correlated with eosinophil infiltration (14, 28), and the degree of this eosinophil infiltration correlated with an unfavorable prognosis (29). Oral squamous cell carcinoma (30) and gastric

FIGURE 5. Rat aortic ring capillary sprouting in response to eotaxin (CCL11). Capillary sprouting occurred from the edge of the ring. A. Negative control. B, CCL11 at 10 nM. C, CCL11 at 10 nM plus anti-human CCL11 at 10 μg/ml. D, bFGF at 60 nM was used as the positive control. Photographs were taken at 4× magnification. E. Angiogenic scores of the sprouting induced by different concentrations of CCL11 ( ), CCL11 together with anti-CCL11 ( ), and bFGF (○). The mean and SEM of three experiments are shown. * p < 0.01; ** p < 0.05.


