Pulmonary Fibroblasts Induce Epithelial Mesenchymal Transition and Some Characteristics of Stem Cells in Non-Small Cell Lung Cancer

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Background. Fibroblasts are key components of the tumor microenvironment. The purpose of this study was to clarify the role of fibroblasts in tumor progression in non-small cell lung cancer (NSCLC).

Methods. Fibroblasts isolated from surgical exploration were co-cultured with human lung adenocarcinoma cell lines. We defined fibroblasts obtained from tumors as cancer associated fibroblasts (CAFs) and those from normal lung tissue as lung normal fibroblasts (LNFs).

Results. Expression levels of myofibroblast markers were higher in CAFs than LNFs within 5 passages in the absence of continuing interaction with carcinoma cells. Thus, we used at least 2 pairs of these CAFs and LNFs in the following experiments; conditioned medium (CM) from fibroblast-induced epithelial mesenchymal transition and acquisition of cancer stem cell-like qualities in lung cancer cells (A549 and NCI-H358), indicating that CM from fibroblasts was biologically active. Furthermore, the concentration of the transforming growth factor (TGF)-β1 was higher in CM from CAFs as compared with that from LNFs, and phenotypic changes of cancer cells by CM from CAFs were greater than those induced by CM from LNFs. These CAF-induced changes were inhibited by addition of the TGF-β inhibitor SB431542. Subcutaneous co-injection of lung cancer cells and CAFs in mice enhanced tumor growth when compared with cancer cells alone, which was attenuated by administration of SB431542.

Conclusions. Fibroblasts were associated with increased malignant potential and the acquisition of stem cell-like properties in NSCLC tumors. Targeting CAFs as a therapeutic strategy against cancer is an intriguing concept that would benefit from further study.

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Lung cancer is the leading cause of cancer death worldwide [1]. Overall prognosis for affected patients is poor due to metastatic disease and lack of curative systemic therapy, underscoring the need for a better understanding of the biologic changes that promote the aggressive neoplastic phenotype [2].

Epithelial to mesenchymal transition (EMT) is a fundamental biologic process during which epithelial cells lose their polarity and change to a mesenchymal phenotype [3]. When cancer cells invade or metastasize, they use a mechanism similar to EMT [4]. Furthermore, some studies have reported a role for EMT in the development of cancer cell resistance to anti non-small cell lung cancer (NSCLC) agents [5–7]. The cancer stem cells (CSC) theory proposes that cancers are maintained by subpopulations of tumor cells that possess progenitor cell characteristics. These cells can initiate tumor formation, differentiate along multipotent pathways, and are relatively resistant to conventional chemotherapy [8–10]. The EMT generates cells with many of the properties of epithelial stem cells [11]. The induction of EMT in primary lung cancer cell line results in the acquisition of mesenchymal profile and in the expression of stem cell markers [12]. Thus, EMT-driven gain of cancer stem cell properties may be associated with aggressiveness and metastatic spread [13].

The tumor microenvironment is a key component of tumor progression, and the invasion of cancer cells into and through the stroma requires EMT [14]. A specific subset of stromal cells, termed cancer associated fibroblasts (CAFs), show morphologic characteristics of both fibroblasts and smooth muscle cells [15]. The CAFs modulate the behavior of adjacent cancer cells by secreting various growth factors and cytokines, thereby promoting tumorigenesis [16]. Tumor-localized CAFs can comprise up to more than half of the tumor mass, and there is active multi-directional communications

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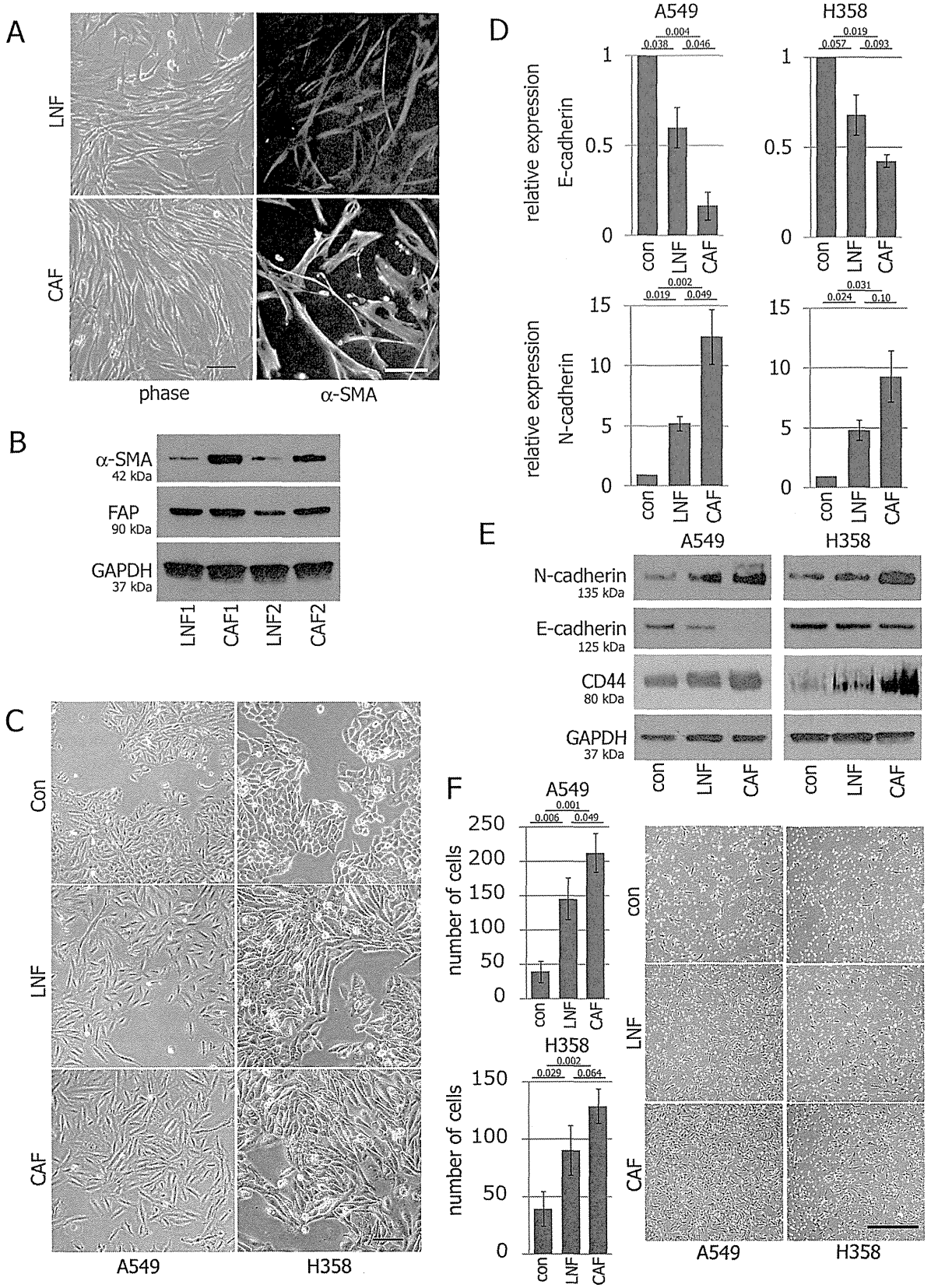
A
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relative expression
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CAF
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N-cadherin
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E-cadherin
125 kDa
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80 kDa
GAPDH
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number of cells

between these coevolving cell types within cancer tissue [17–19].

In order to identify new targets for prevention of metastasis, it is important to understand the molecular mechanisms that drive EMT. Therefore, the goal of the present study was to clarify the role of CAFs in the induction of EMT and CSC of NSCLC cells.

Material and Methods

Cell Culture and Materials

A549 cells and NCI-H358 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in Roswell Park Memorial Institute 1640 with 10% fetal bovine serum (FBS). Antibodies for western blotting and immunofluorescence were as follows: anti-alpha-smooth muscle actin (α-SMA) pAb (catalog number ab5694; Abcam, Tokyo, Japan), anti-fibroblast activating protein pAb (Abcam, ab53066), anti-glyceraldehyde-3-phosphate dehydrogenase mAb (G9545; Sigma-Aldrich, St. Louis, MO), anti-N-cadherin (sc-59987; Santa Cruz Biotechnology, Inc, Dallas, TX), anti-E-cadherin mAb (M106; Takara, Otsu, Shiga, Japan), and anti-CD44 mAb (156-3C11; Cell Signaling Technology, Beverly, MA). Antibodies for the immunohistochemistry experiments were as follows: anti-E-cadherin mAb (M3612; Dako, Carpinteria, CA), anti-CD44 mAb (156-3C11; Cell Signaling), and Ki67 (M7240; Dako). Cisplatin was purchased from Sigma (catalog# 479306, St. Louis, MO), and transforming growth factor (TGF)-β1 was from R&D Systems (240-B; Minneapolis, MO), and SB431542 was from Tocris Bioscience (1614; Ellisville, MO).

Isolation and Primary Culture of Fibroblasts

Fibroblasts were isolated from surgical explanation. We defined the fibroblasts from the non-necrotic part of the tumors as CAFs and those from normal lung as LNFs. Briefly, cancerous tissue or normal lung far from cancerous tissue were obtained aseptically from 5 patients with NSCLC undergoing pulmonary resection after the Institutional Review Board for Clinical Research at Osaka University Hospital approved our study protocol and written informed consent for surgical intervention was obtained from each patient. Tissues were digested for 6 hours in 1 mg/mL collagenase I (Sigma), and cells were plated in Dulbecco's Modified Eagles medium (DMEM) containing 10% FBS. Two weeks later, proliferated cells were selected using anti-Fibroblast MicroBeads and MACS Columns (Miltenyi Biotec, Auburn, CA), confirmed by positive staining for vimentin and negative staining for pan-cytokeratin, and continued to culture as fibroblasts. Conditioned media (CM) were obtained from 48-hour serum-starved cells (6 mL DMEM for fibroblasts plated semi-confluent to a 10-cm dish) and used freshly. All in vitro experiments were performed in triplicate using 2 pairs of primary cultured CAFs and LNFs.

Real-Time RT-PCR

Total RNA was isolated from cells treated with or without CM from fibroblasts for 24 hours using RNeasy Mini Kit (Qiagen, Tokyo, Japan). Real-time RT-PCR (E-cadherin, Hs00170423_m1; N-cadherin, Hs00169953_m1 [Applied Biosystems, Tokyo, Japan]) was performed using a CFX96 system (BioRad, Tokyo, Japan) and relative expression levels were calculated by the comparative Ct method.

Detergent Extraction, SDS-PAGE, and Immunoblots

Monolayers of cultured cells, treated with or without CM from fibroblasts for 48 hours, were extracted with RIPA buffer (Cell Signaling, catalog# 9806). Cell extracts were resolved by SDS-PAGE [sodium dodecyl sulfate polyacrylamide gel electrophoresis] and immunoblotted as described [20].

Trans-Well Motility Assays

We plated 5×10^5 cells in the upper chamber of polyethylene terephthalate membranes (pore size 8 μm; Becton Dickinson, Franklin Lakes, NJ). The number of cells on the lower side of the filter was counted under microscope after 8 hours incubation.

Spheroids Culture

Cells were cultured in low adherent 35-mm dishes (Corning, Corning, NY) under serum-free condition for 21 days [21]. Briefly, 1×10^3 cells were suspended in 6 mL of DMEM or CM from CAFs or LNFs, supplemented with 10 ng/mL basic fibroblast growth factor. Spheroid bodies were harvested every 5 days and re-cultured with fresh media. To inhibit TGF-β signaling, SB431542 was added to CM. Spheres with a diameter over 50 μm were counted on an inverted microscope.

Cell Viability Assay

For quantitative viability assays, Cell Proliferation Kit I (MTT; 3-[4.5 dimethylthiazol 2-yl]-2,5-diphenyltetrazolium

Fig 1. (A) We defined fibroblasts obtained from tumors as cancer associated fibroblasts (CAFs) and those from normal lung tissue as lung normal fibroblasts (LNFs). Phase contrast pictures of LNFs and CAFs were taken (left panels). Scale bar: 100 μm. The LNFs and CAFs were stained with α- smooth muscle actin (SMA). Scale bar: 50 μm. (B) Two pairs of LNFs and CAFs were analyzed on Western blot for α-SMA, fibroblast activating protein (FAP), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. (C) A549 and NCI-H358 cells were treated with Dulbecco's Modified Eagles medium (DMEM) as a control, Con) or conditioned medium (CM) from LNFs and CAFs for 2 days. Phase contrast images were taken. Scale bar: 100 μm. (D) A549 and NCI-H358 cells were treated with and without CM from CAFs or LNFs for 24 hours. Cells were analyzed on real-time reverse transcriptase-polymerase chain reaction to detect E-cadherin and N-cadherin. The results are shown as mean ± SD. Significance was tested with the Mann-Whitney test and exact p values are shown. (E) A549 and NCI-H358 cells treated with DMEM (con) or CM from LNFs and CAFs for 2 days were analyzed on Western blot for N-cadherin, E-cadherin, CD44, and GAPDH. (F) Motility assays were performed and images were obtained. Cells transversing the filter were counted. Scale bar: 100 μm. Columns represent mean ± SD and p values are shown.

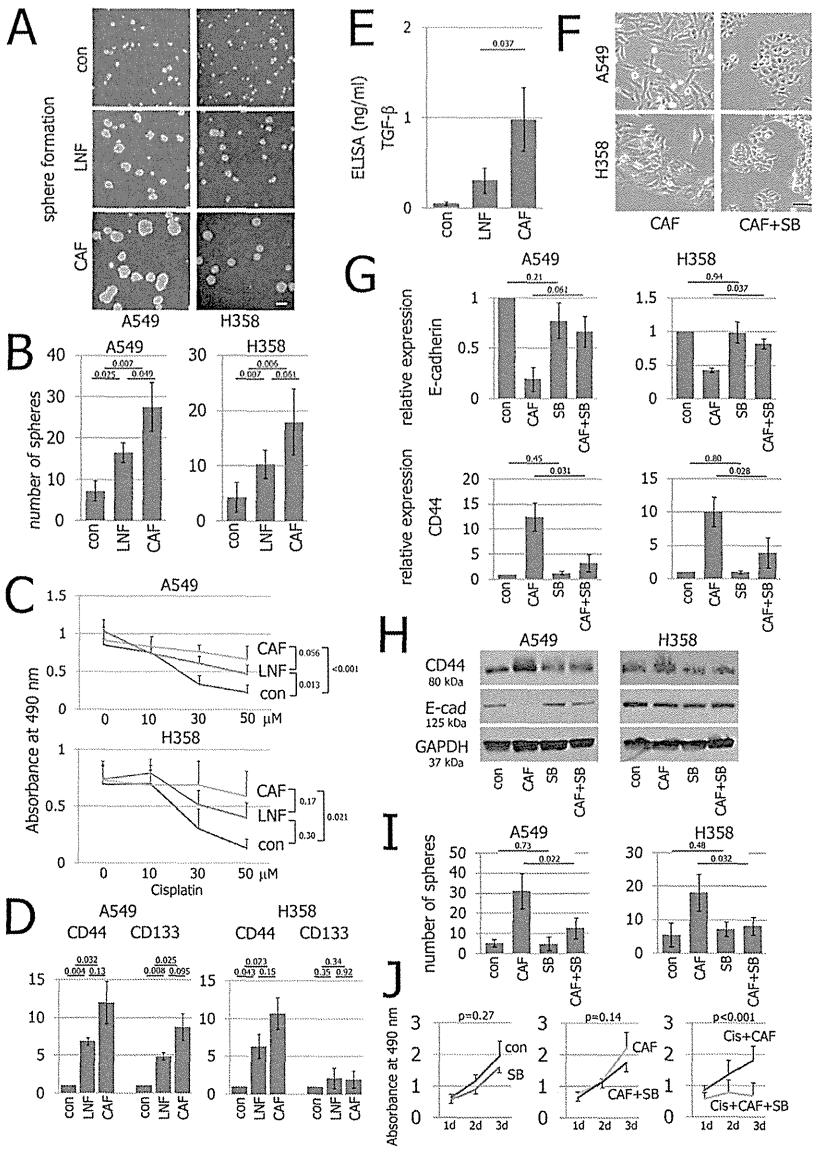


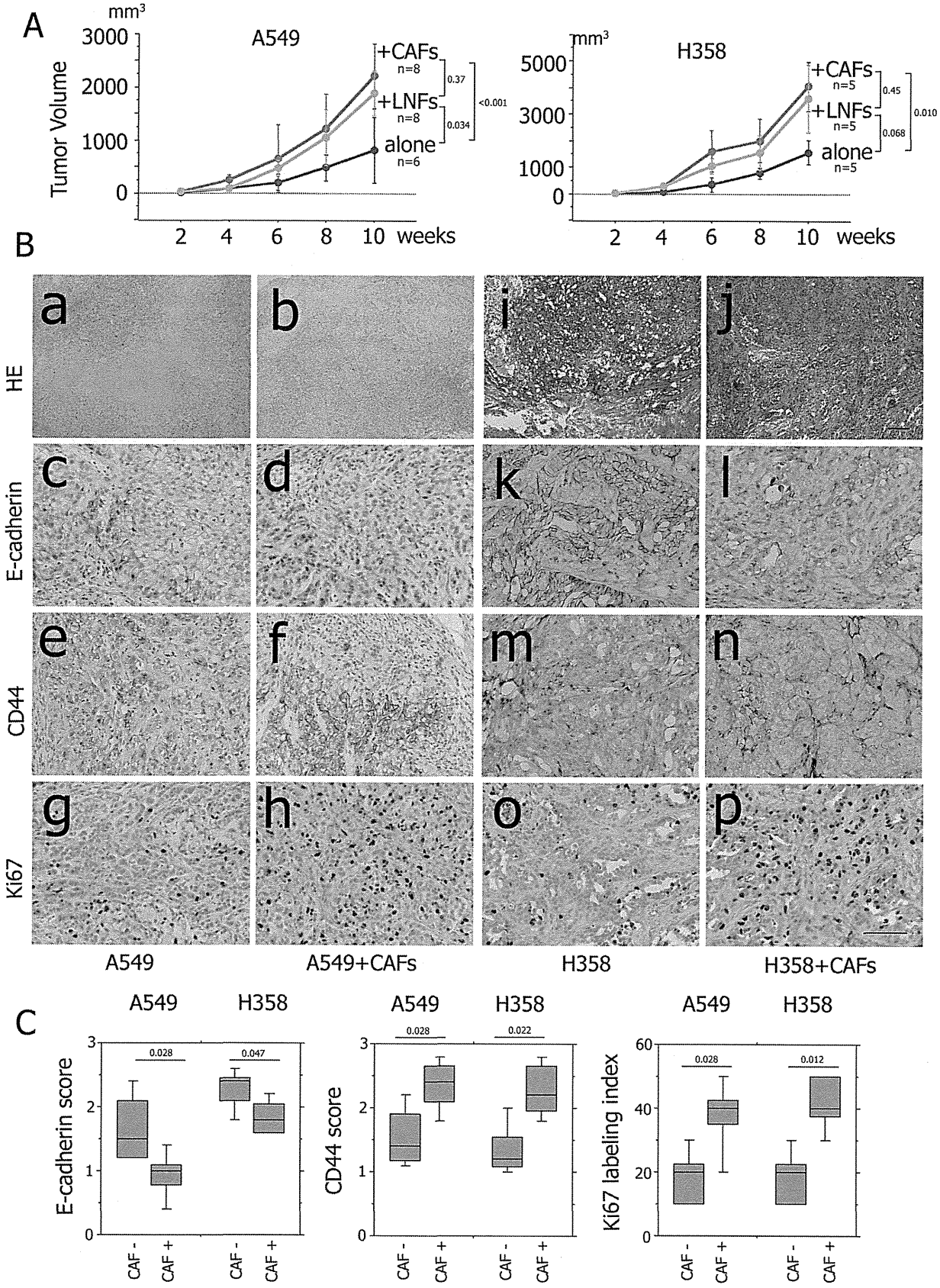
Fig 2. (A) A549 and NCI-H358 cells were grown under spheroid culture condition with or without conditioned medium (CM) from cancer associated fibroblasts (CAFs) or lung normal fibroblasts (LNFs). Floating spheroid bodies were photographed. Scale bar: 50 μm. (B) Floating spheroid bodies were counted and the results are shown as mean ± SD with exact p *values. (C) A549 and NCI-H358 cells were treated with cisplatin with or without CM from CAFs or LNFs. Proliferation of cells 2 days after seeding was quantified and significance was tested with the repeated measures analysis of variance. (D) A549 and NCI-H358 cells were treated with and without CM from CAFs or LNFs for 24 hours. The real-time reverse transcriptase-polymerase chain reaction (RT-PCR) performed to detect CD44 and CD133. (E) CM was collected from 2 pairs of LNFs and CAFs and quantification of transforming growth factor (TGF)-β1 was assessed by enzyme-linked immunosorbent assay; Con, control (culture media for fibroblasts). (F) A549 and NCI-H358 cells were treated with CM from CAFs for 2 days with or without the TGF-β inhibitor; SB431542. Phase contrast images were taken; scale bar: 100 μm. (G) A549 and NCI-H358 cells were cultured in CM from CAFs with or without SB431542 for 24 hours. The real-time RT-PCR performed to detect CD44 and CD133. (H) A549 and NCI-H358 cells cultured in CM from CAFs with or without SB431542 for 48 hours were analyzed on Western blot for CD44, E-cadherin, and glyceraldehyde-3-phosphate dehydrogenase. (I) A549 and NCI-H358 cells were grown under spheroid culture condition with CM from CAFs including SB431542. Floating spheroid bodies were counted and the results are shown as mean ± SD. (J) Proliferation of A549 cells was quantified by MTT assay. A549 cells were cultured in Dulbecco's Modified Eagles medium (DMEM) (as a control, con), DMEM with SB431542 (10 μM, SB), CM from CAF, CM with SB431542 (10 μM, CAF + SB) for 3 days (1d = 1 day; 2d, 2 days; 3d = 3 days after treatment). Cells were also treated with CM including cisplatin (30 μM, Cis + CAF) and CM including both cisplatin (30 μM) and SB431542 (10 μM, Cis + CAF + SB).*

bromide) was obtained from Roche Applied Science (Mannheim, Germany). Cells were plated in 96-well plates (1×10^4 cells/50 μL serum free media for each well). Fifty μL of serum free media, CM from CAFs, or CM from LNFs were added to each well. The cells were treated according to the manufacture's protocol and absorbance was measured at 490 nm using a microplate reader (BioRad) after indicated days. Cisplatin or SB431542 was added at various concentrations.

Enzyme-Linked Immunosorbent Assay (ELISA) for TGF-β1

Around 1×10^6 cells were plated in 10-cm dishes, and cells were fed with serum-free DMEM (6 mL) next day.

Fig 3. (A) Tumor volumes (mean ± SD) after subcutaneous injection of A549 or NCI-H358 cells with or without cancer associated fibroblasts (CAFs) or lung normal fibroblasts (LNFs) into nude mice were plotted and significance was tested with the repeated measures analysis of variance. Exact p *values are shown. (B). Panels a to p show representative findings from primary tumor obtained from mice. Scale bar: 100 μm. (C) Expression of E-cadherin and CD44 were scored in a semiquantitative manner and Ki-67 labeling index was calculated. Bars for the box extend from the 25th to 75th percentile of the data and the line in the middle represents the median. The upper and lower bars represent the distance from the 10th to 90th percentile from the median. (HE = hematoxylin and eosin.)*


A
mm³
3000
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Tumor Volume
A549
+CAFs
n=8
+LNFs
n=8
alone
n=6
0.37
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<0.001
2 4 6 8 10 weeks
mm³
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3000
1000
0
H358
+CAFs
n=5
+LNFs
n=5
alone
n=5
0.45
0.068
0.010
2 4 6 8 10 weeks
B
HE
E-cadherin
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A549
A549+CAFs
H358
H358+CAFs
C
A549
H358
E-cadherin score
0.028
0.047
CAF -
CAF +
A549
H358
CD44 score
0.028
0.022
CAF -
CAF +
A549
H358
Ki67 labeling index
0.028
0.012
CAF -
CAF +

The CM was collected after 48 hours and quantification of TGF-β1 was assessed by ELISA according to the manufacture's protocol (human Quantikine; R&D System, Minneapolis, MN).

Animal Studies

All manipulations were done in accordance with protocols approved by the Osaka University Institute Animal Care Committee. The 1×10^6 tumor cells (A549 or NCI-H358 cells) mixed with or without 1×10^5 CAFs or LNFs (2 pairs of primary cultured CAFs and LNFs for A549 cells and 1 pair of those for H358 cells [A549 alone, n = 6; A549 + CAFs, n = 8; A549 + LNFs, n = 8; H358 alone, n = 4; H358 + CAFs, n = 4; H358 + LNFs, n = 4]) were injected subcutaneously into the back of 4-week-old nude mice. Tumor volume was calculated according to the formula: tumor volume (mm^3) = $d^2 \times D/2$, where d and D were the shortest and the longest diameters, respectively. The SB431542 was injected intraperitoneally at 10 mg/kg (once per day, 5 times a week for 2 weeks) 1 week after co-injection (1×10^6 A549 cells with or without 1×10^5 CAFs, n = 5 for each group) according to protocols described in previous reports [22, 23].

Immunohistochemistry

Immunohistochemistry was performed as previously described [7]. All sections stained with E-cadherin and CD44 were scored in a semiquantitative manner classified as 0 (no staining), +1 (weak staining), +2 (moderate staining), or +3 (strong staining) in intracellular compartments of carcinoma cells [24]. The Ki-67 labeling index (labeling frequency %) was calculated by the following formula: [number of positive nuclei/total number of represented cells] ×100. All immunohistochemistry results were quantified by counting at least more than 200 tumor cells in randomly selected 5 areas in each specimen.

Statistical Design and Data Analysis

A χ^2 test, Mann-Whitney *U* test, or repeated measures analysis of variance was used to compare the results. All statistical analyses were performed using StatView version 5.0 for Windows (Abacus Concepts, Berkeley, CA).

Results

NSCLC Cell Lines Undergo EMT in Response to Conditioned Medium From Fibroblasts

Primary cultured CAFs and LNFs each expressed α-SMA and fibroblast activating protein, with the levels greater in CAFs than LNFs even after 5 passages (Fig 1A, 1B). Conditioned medium from both also induced EMT in A549 and NCI-H358 cells, which became more spindle shaped (Fig 1C). Furthermore, the expression of E-cadherin decreased and that of N-cadherin increased in cancer cells (Fig 1D, 1E). These changes in EMT markers were significantly greater in A549 cells treated with CM from CAFs as compared with those treated with CM from LNFs, while migration induced by CAFs was significantly greater than that induced by LNFs in A549 cells (Fig 1F). These findings suggest that paracrine interplay between fibroblasts and cancer cells leads to EMT in cancer cells. In addition, they indicate that CAFs can maintain the phenotypic properties of myofibroblasts even in the absence of continuing interaction with carcinoma cells.

Spheroid Formation Ability and Resistance to Treatment by NSCLC Cell Lines in Response to CM From Fibroblasts

When cultured with CM from fibroblasts, both A549 and NCI-H358 cells gave rise to significantly greater spheroid body formation as compared with cells cultured without CM (Fig 2A, 2B). Furthermore, CM-treated cells showed a significant increase in cell viability in response to cisplatin when compared with untreated control cells (Fig 2C).

CAF-Mediated EMT Promoted Generation of Cancer Stem Cells

We evaluated changes in the CSC markers CD44 and CD133. The CM from CAFs increased the expression of CD44 to a greater degree than that seen after treatment with CM from LNFs (Fig 2D). The SDS-PAGE results also showed that the expression of CD44 was increased to a greater degree in cancer cells treated with CM from CAFs than in those treated with CM from LNFs (Fig 1E).

Blocking TGF-β Signaling Inhibited Phenotypic Changes Associated With EMT and Stem Cell-Like Qualities

Transforming growth factor-β is a strong inducer of EMT in epithelial cells. The ELISA findings showed that the concentration of TGF-β1 was greater in CM from CAFs as compared with that from LNFs (Fig 2E). Thus, we blocked TGF-β signaling to clarify the role of TGF-β in CAF-induced EMT. The CAF CM-driven phenotypic changes were inhibited by addition of the TGF-β inhibitor SB431542 to CM (Fig 2F), while down-regulation of E-cadherin and increased CD44 expression (Fig 2G, 2H), and spheroid formation ability induced by CM from CAFs (Fig 2I) were significantly inhibited by SB431542. Although TGF-β inhibition did not change the growth of cancer cells after 2 days, it attenuated the resistance to chemotherapy in vitro (Fig 2J). Taken together, these results suggest that TGF-β signaling may mediate EMT and maintenance of stem cell-like qualities induced by CAFs.

CAF-Mediated Tumor Formation of Human Lung Adenocarcinoma Cell Lines

Although cancer cells co-cultured with fibroblasts did not show an increase in cell proliferation (Fig 2J, compare left panel with middle panel, $p = 0.74$), subcutaneous co-injection of each lung cancer cell line with human CAFs or LNFs into mice resulted in a high rate of tumor formation, as compared with injection of cancer cells alone (Fig 3A). There was no difference between co-injection with cancer cells and CAFs and that with LNFs. We also compared specimens obtained from mice injected with cancer cells alone with those co-injected with cancer cells and CAFs. Hematoxylin and eosin

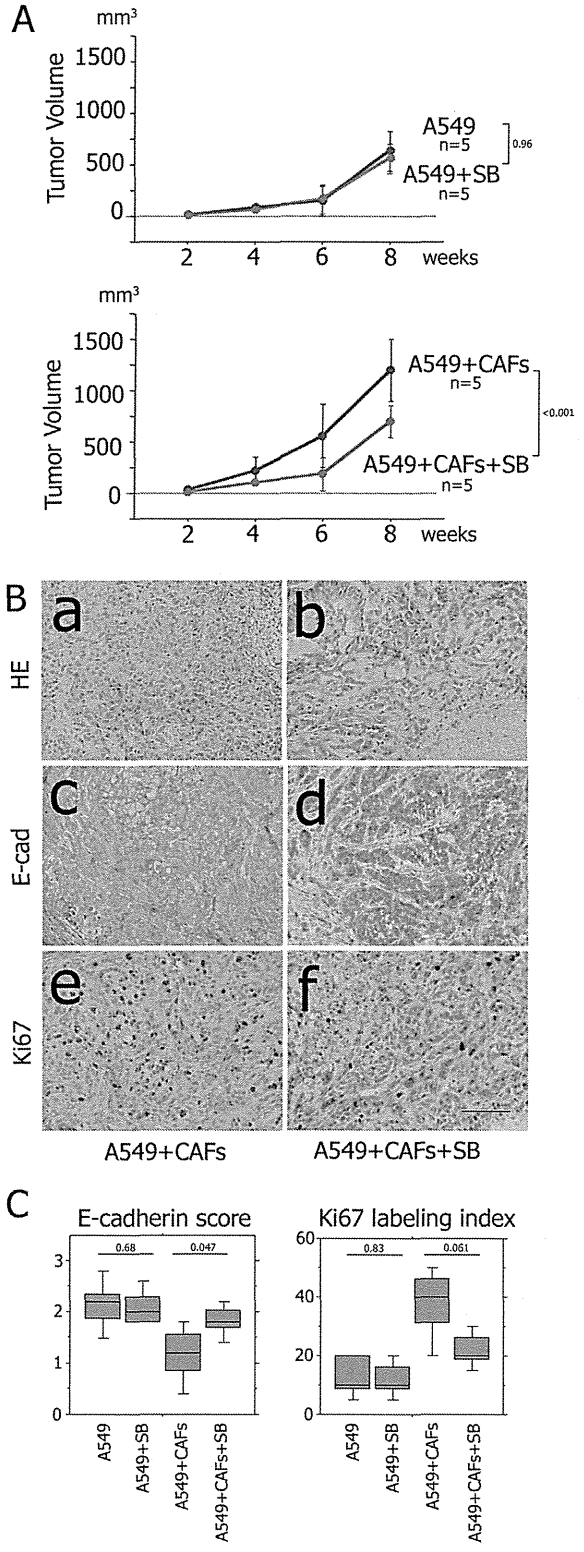

Fig 4. (A) The transforming growth factor (TGF)-β inhibitor, SB431542, was injected intraperitoneally after subcutaneous injection of A549 cells, with or without cancer associated fibroblasts (CAFs), into nude mice. Tumor volumes (mean ± SD) were plotted and significance was tested with the repeated measures analysis of variance. Exact p values are shown. (B) Panels a through f show representative findings from primary tumor obtained from mice treated with or without the TGF-β inhibitor, SB431542. Panels a, c, e: mice with A549 in combination with CAFs; panels b, d, f: mice treated with the TGF-β inhibitor SB431542 (SB) after injection with A549 in combination with CAFs. (C) Expression of E-cadherin was scored and Ki-67 labeling index was calculated, and the results are shown in the same way as Figure 3C.

staining revealed both necrosis and apoptotic cell death in the middle of tumors from mice injected with A549 or H358 cells alone (Fig 3B). On the other hand, E-cadherin and CD44 expressions were changed in tumors from mice that underwent co-injection with cancer cells and CAFs as compared with those with cancer cells alone. We compared these findings using a semiquantification technique, which showed that the expression of E-cadherin was lower and that of CD44 higher in cancer cells injected with CAFs as compared with cancer cells alone (Fig 3C). Furthermore, the Ki-67 labeling index was also higher in tumors from mice injected cancer cells and CAFs as compared with cancer cells alone. These results suggest that co-injection with A549 and CAFs induced EMT and stem cell-like qualities in cancer cells, resulting in rapid tumor growth in vivo.

When compound SB431542 was injected in an intraperitoneal manner, tumor formation enhanced by co-injection with CAFs was attenuated, whereas that compound did not affect tumor growth in mice injected with A549 alone (Fig 4A). Furthermore, the expression of E-cadherin was increased and Ki-67 labeling index was decreased in tumors obtained from mice treated with SB431542 (Fig 4B, 4C).

Comment

In the present study we found that CM from fibroblasts induced EMT in NSCLC cells, while those cells also acquired characteristics of stem cell-like qualities. The EMT changes induced by CAFs in A549 cells were greater than those induced by LNFs, indicating that CAFs may have been more activated in our experimental system as compared with LNFs. Navab and colleagues [25] reported that CAFs were activated to a greater degree than LNFs by showing α-SMA expression and collagen gel contraction. Together, these findings suggest that CAFs play a more crucial role in tumor homeostasis, progression, and maintenance of stem cell-like qualities in cancer cells as compared with normal fibroblasts. However, it is difficult to compare CAFs with LNFs in a rigorous manner. One reason is that the present fibroblasts were isolated from heterogeneous tissues obtained from patients with different backgrounds. Furthermore, while fibroblasts are in an inactive and quiescent state in normal tissues, isolated fibroblasts may become activated and release mediators when cultured with FBS or co-cultured with cancer cells. We found that primary cultured CAFs and LNFs each expressed α-SMA and fibroblast activating protein,

though those levels were greater in CAFs than LNFs after 5 passages. Therefore, the cells used in the present in vitro experiments were collected within the limit of 5 passages. We found that co-injection of each lung cancer cell line with human fibroblasts into mice resulted in a high rate of tumor formation. A previous report [25] noted that CAFs demonstrated a greater ability to enhance the tumorigenicity of lung cancer cell lines in vivo, while co-injection with CAFs increased tumor formation in a manner similar to that seen when co-injected with LNFs in the present experimental system. It is possible that co-injection of LNFs with cancer cells may stimulate LNFs in vivo and change their characteristics in a manner similar to CAFs. Although the marker profile of lung cancer stem cells remains to be fully elucidated, recent findings suggest that CD44 or CD133 may be an effective lung cancer stem cell marker [26, 27]. In addition, CD44 has been associated with a number of signaling cascades that mediate tumor progression and resistance to chemotherapy [28, 29]. Cancer cells in the present study showed increased levels of these CSC markers, supporting the notion that fibroblasts in the tumor stroma maintain cancer stem cell-like properties.

Phenotypic changes associated with EMT and stem cell-like qualities were inhibited by addition of the TGF-β inhibitor SB431542, indicating that TGF-β signaling plays a major role in fibroblast-induced changes. The TGF-β is expressed by both cancer and stromal cells, and establishes interactive pathways in the cross-talk between them [2]. In the efferent pathway cancer cells trigger a reactive response in the stroma, while in the afferent pathway cells responding to modified stromal cells in the surrounding microenvironment have effects on cancer cell response [30, 31]. These interactive pathways, which become established during cross-talk between cancer and stromal cells, may form a malignant signaling cycle for cancer progression. Because CAFs are genetically stable and less likely to develop drug resistance [15], targeting them as a therapeutic strategy against cancer is an intriguing concept that would benefit from further study.

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ABTS Requirements for the 10-Year Milestone for Maintenance of Certification

Diplomates of the American Board of Thoracic Surgery (ABTS) who plan to participate in the 10-Year Milestone for the Maintenance of Certification (MOC) process as Certified-Active must hold an unrestricted medical license in the locale of their practice and privileges in a hospital accredited by the JCAHO (or other organization recognized by the ABTS). In addition, a valid ABTS certificate is an absolute requirement for entrance into the MOC process. If your certificate has expired, the only pathway for renewal of a certificate is to take and pass the Part I (written) and the Part II (oral) certifying examinations.

The CME requirements are 150 Category I credits over a five-year period. At least half of these CME hours need to be in the broad area of thoracic surgery. Category II credits are not accepted. Interested individuals should refer to the Board's website (www.abts.org) for a complete description of acceptable CME credits.

Diplomates will be required to take and pass a secured exam after their application has been approved. Taking SESATS in lieu of the secured exam is not an option. The secured exam is administered over a two-week period in September of every year at Pearson Vue Testing Centers, which are located nationwide. Diplomates will have the opportunity to select the day and location of their exam. For the dates of the next MOC exam, visit the Board's web site at www.abts.org.

Starting on July 1, 2014, the ABTS will require its Diplomates to participate in an outcomes database as fulfillment of Part IV (Performance in Practice) for the 10-year Milestone of Maintenance of Certification (MOC). For a list of approved outcomes databases or for more information on how to have a database approved by the Board, visit the Board's website at www.abts.org. Participation in the Professional Portfolio will no longer be accepted as fulfillment of MOC Part IV after July 1, 2014.

Diplomates may apply for MOC in the year their certificate expires or, if they wish to do so, they may apply up to two years before it expires. However, the new certificate will be dated 10 years from the date of expiration of their original certificate or most recent MOC certificate. In other words, going through the MOC process early does not alter the 10-year validation. Diplomates certified prior to 1976 (the year that time-limited certificates were initiated) are also required to participate in MOC if they wish to maintain valid certificates.

The deadline for submitting an application for 10-year Milestone of MOC is March 15 of every year. Information outlining the rules, requirements, and dates for MOC in thoracic surgery is available on the Board's website at www.abts.org. For additional information, please contact the American Board of Thoracic Surgery, 633 N St. Clair St, Ste 2320, Chicago, IL 60611; telephone (312) 202-5900; fax (312) 202-5960; e-mail: info@abts.org.

Immunological functions of the neuropilins and plexins as receptors for semaphorins

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Abstract | Semaphorins were originally identified as axon-guidance molecules that function during neuronal development. However, cumulative evidence indicates that semaphorins also participate in immune responses, both physiological and pathological, and they are now considered to be potential diagnostic and/or therapeutic targets for a range of diseases. The primary receptors for semaphorins are neuropilins and plexins, which have cell type-specific patterns of expression and are involved in multiple signalling responses. In this Review, we focus on the roles of neuropilin 1 (NRP1) and plexins in the regulation of the immune system, and we summarize recent advances in our understanding of their pathological implications.

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Semaphorins were originally identified as axonal growth cone-collapsing proteins that are required to direct neuronal axons to their appropriate targets[1]. Since the 1990s, when their biological functions were first reported, more than 20 members of the semaphorin family have been found in vertebrates[2]. Two groups of proteins, the neuropilins (NRP1 and NRP2) and plexins (plexins A1, A2, A3, A4, B1, B2, B3, C1 and D1), have been identified as the main semaphorin receptors[3–5]. Secreted semaphorins (known as class 3 semaphorins) generally require NRPs as obligate co-receptors to interact with plexins, whereas most membrane-associated semaphorins (known as classes 4, 5, 6 and 7 semaphorins) directly bind to plexins (BOX 1; FIG. 1).

Recent findings have elucidated distinctive mechanistic aspects of these semaphorin receptors; for example, NRP1 is a regulatory T (T_{Reg}) cell marker[6–9], and there is crosstalk between plexin-mediated signalling and other signalling pathways, such as WNT- and insulin-like growth factor 1 (IGF1)-mediated signalling pathways[10,11]. In addition, the accumulated evidence has established that semaphorins and their receptors are involved in many processes beyond axon guidance, including cardiovascular development and growth[12,13], tumour progression, metastasis and suppression[14–16], osteoclastogenesis[10,11,17], homeostasis of the retina[18] and immune cell regulation[19–25]. Semaphorins and their receptors have crucial roles in various phases of physiological and pathological immune responses; these proteins constitute a family of immunoregulatory molecules that we refer to as immune semaphorins[26]. From a clinical point of view, semaphorins and their receptors have been implicated in various human diseases, including tumorigenesis[5], tumour metastasis[27], neurodegenerative diseases[28] and immune disorders[29].

In light of these recent advances, semaphorins, as well as their receptors and their related signalling molecules, are considered to be potential diagnostic and therapeutic targets for various human diseases, including autoimmunity and allergy. In addition, recent protein structural studies have clearly determined the molecular basis for their ligand–receptor interactions, which provides powerful information to use to develop semaphorin-targeted therapies. In this Review, we discuss our rapidly increasing knowledge of the roles of semaphorin receptors in mice and, where possible, in humans (TABLE 1) to better understand their physiological and pathological implications.

Neuropilin 1

NRP1 and NRP2 are highly conserved transmembrane proteins that were originally identified as neuronal adhesion molecules that function during neuronal development[30]. NRPs were subsequently identified as neuronal receptors for secreted class 3 semaphorins such as semaphorin 3A (SEMA3A)[31,32]. As NRPs have short cytoplasmic domains (~40 amino acid residues in length) (FIG. 2), their signals are generally mediated through interacting co-receptors such as plexins[33,34]; for example, after binding to class 3 semaphorins, NRPs then associate with class A plexins[33,34]. NRPs also function as co-receptors for several other receptor systems that are involved in

Box 1 | **Semaphorins and their receptors**

The semaphorin family comprises a large number of phylogenetically conserved proteins that are structurally characterized by the Sema domain in their extracellular regions. The Sema domain has a seven-blade β-propeller structure containing sites for dimerization and binding to semaphorin receptors. On the basis of their carboxy-terminal structural features, semaphorins have been subdivided into eight classes. Members of class 3 are secreted, whereas the other vertebrate semaphorins are membrane associated (classes 4, 5 and 6 are transmembrane proteins, whereas class 7 proteins are membrane bound) and can be cleaved from the cell surface in certain conditions[3,5]. Of note, classes 1 and 2 are encoded by invertebrates. Class 8 semaphorins are virally encoded.

Generally, membrane-bound semaphorins (class 4–7) directly bind to plexins. By contrast, secreted semaphorins (class 3) require neuropilins (NRP1 and NRP2) as direct binding co-receptors to enable binding to plexins. However, a growing body of evidence has shown that semaphorin–receptor interactions are more complex than this; for example, semaphorin 3E (SEMA3E) — a secreted semaphorin — directly binds to plexin D1, without NRPs. In both the nervous and immune systems, SEMA7A associates with integrins in addition to plexin C1. In the immune system specifically, SEMA4A and SEMA4D use T cell immunoglobulin and mucin domain-containing protein 2 (TIM2) and CD72, respectively, as a receptor in addition to members of the plexin B family.

In vertebrates, the plexin family consists of nine members, which are canonical semaphorin receptors involved in mediating cytoplasmic signals. In the nervous system, plexin-mediated signals regulate the activities of GTPases and of cytoplasmic or receptor-type protein kinases, as well as regulating integrin-mediated attachment. Plexins can associate with different co-receptors to confer pleiotropic functions on semaphorins; for example, in heart morphogenesis, plexin A1 forms heterodimers with the tyrosine kinase receptors off-track (OTK) and vascular endothelial growth factor receptor 2 (VEGFR2), whereas during osteoclastogenesis, plexin A1 forms receptor complexes with triggering receptor expressed on myeloid cells 2 (TREM2)– DNAX activation protein 12 (DAP12) and NRP1. Plexin B1 associates with the receptor tyrosine kinases MET and ERBB2 to induce the invasive growth of epithelial cells. Thus, semaphorins can trigger multiple signalling cascades to carry out their diverse biological activities.

development, immunity and cancer[35]; for example, NRP1 is a receptor for vascular endothelial growth factor (VEGF) family members (including the splice variant $VEGF_{165}$), which are expressed by endothelial cells and tumour cells[36], as well as for transforming growth factor-β1 (TGFβ1)[37]. The extracellular domains of NRPs have been shown to have adhesive properties; therefore, careful and critical evaluation of the interactions between NRP1 and other receptors and ligands will be required to definitively determine the roles of NRP1. In addition, it has been reported that the short cytoplasmic domain of NRP1 has a role in integrin functions and VEGF signalling[38–40]. The immunological analysis of NRP2, which has a similar structure to NRP1, is still in its infancy. In this Review, we describe what is known about the role of NRP1 in the immune system.

Axonal growth-cone collapsing proteins
Molecules that induce the loss of motile activity and the cessation of advance of growth cones (the growing tips of axons). Such an axonal guidance process is important to establish connections between pathways in the developing nervous system.

$VEGF_{165}$
The most active and abundant splice variant of vascular endothelial growth factor (VEGF). It functions as a growth factor in angiogenesis, vasculogenesis and endothelial cell growth.

Experimental autoimmune encephalomyelitis
(EAE). A widely used animal model for studies of multiple sclerosis, which is an inflammatory demyelinating disease of the central nervous system (CNS). It is induced by stimulating an immune response directed against CNS antigens.

Expression in the immune system. In recent years, it has become clear that NRP1 has a role in the immune response. During the search for human dendritic cell (DC) markers, NRP1 was identified as blood DC antigen 4 (BDCA4; also known as CLEC4C and CD304), which is expressed by plasmacytoid DCs (pDCs)[41]. pDCs express Toll-like receptors (TLRs) and thereby recognize viral nucleic acids, which results in the production of high levels of type I interferons (IFNs)[42]. Therefore, the functional role of NRP1 in pDCs has been investigated in the context of viral infection. Incubation of pDCs with an NRP1-specific antibody blocks the induction of IFNα production by viral infection or nucleic acids[43]. However, the mechanisms that contribute to this phenotype remain unclear.

In the human thymus, NRP1 is expressed on the cell surface of developing T cells; thus, NRP1 expression can be detected in both the cortex and the medulla of the thymus[44]. NRP1 expression has also been observed in thymic epithelial cells (TECs) and DCs, which indicates that it might be involved in thymocyte development. In addition, *in vitro* co-culture experiments indicate that NRP1 forms homophilic interactions at the cell–cell contacts between T cells and DCs, which suggests that it contributes to the primary immune response in the lymph nodes[45]. However, it remains unclear how and to what extent such homophilic interactions are physiologically relevant to immune responses.

Inhibitory functions: a regulatory T cell marker? The functions of NRP1 in the immune system have been linked to immune inhibition. NRP1 is a specific marker for mouse $CD4^+CD25^+$ T_{Reg} cells, although it is poorly expressed by human T cells. Microarray profiling showed that *Nrp1* is a forkhead box P3 (FOXP3)-inducible gene, as are *CD25* (also known as *IL2RA*), glucocorticoid-induced TNF receptor-related protein (*GITR*; also known as *TNFRSF18*) and cytotoxic T lymphocyte antigen 4 (*CTLA4*)[6,46]. NRP1 expressed by T_{Reg} cells might help to increase the contact time between T_{Reg} cells and DCs through its homophilic interaction with DC-expressed NRP1, which might thereby stabilize the interaction between these cells and prevent naive T cells from interacting with DCs[46]. Therefore, NRP1 seems to contribute to the negative regulation of immune responses by increasing T_{Reg} cell activities. A growing body of evidence supports the idea that NRP1 is functionally relevant in T_{Reg} cell-mediated immune suppression. First, the lack of NRP1 on $CD4^+$ T cells results in increased severity of experimental autoimmune encephalomyelitis (EAE). In addition, T cells from mice with a conditional knockout of *Nrp1* show preferential commitment to the T helper 17 (T_H17) cell lineage and decreased T_{Reg} cell functions[47]. Second, $NRP1^+$ T_{Reg} cells accumulate in the draining lymph nodes of metastatic tumours, which suggests that NRP1 has a

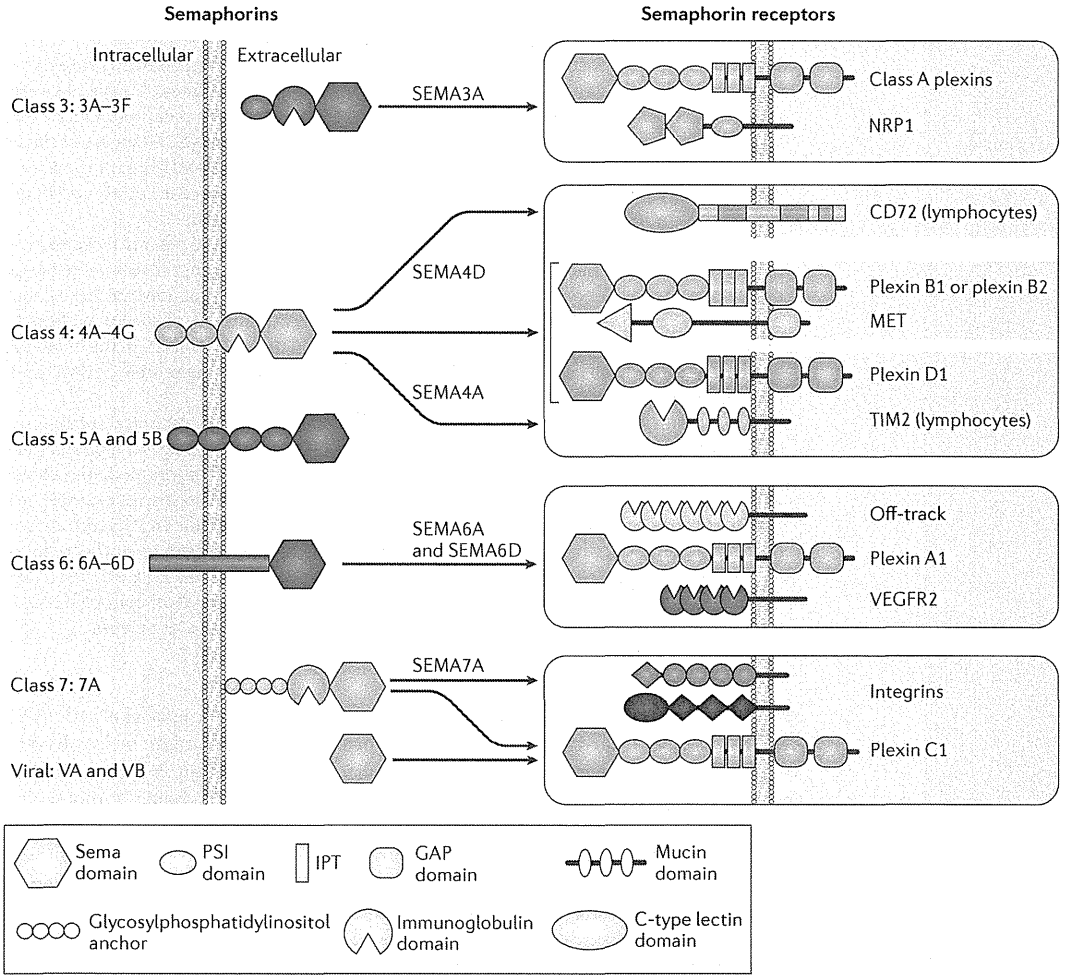

Figure 1 | **The structure of and interactions between semaphorins and their receptors.** Semaphorins are characterized by an extracellular amino-terminal Sema domain followed by one or more cysteine-rich PSI (plexin, semaphorin and integrin) domains. Plexins, which are the most common receptors of semaphorins, consist of an N-terminal Sema domain, followed by a combination of PSI domains and IPTs (immunoglobulin domains shared by plexins and transcription factors) in their extracellular regions. Crystallization studies have shown that semaphorins and plexins interact through their Sema domains. Secreted-type class 3 semaphorins typically require the co-receptor neuropilin 1 (NRP1) to interact with the class A plexin receptor complex. However, semaphorin 3E (SEMA3E) can bind to plexin D1 in a NRP-independent manner (not shown). Membrane-associated class 4 semaphorins bind to class B and class D plexin receptors. In lymphocytes, SEMA4A also binds T cell immunoglobulin and mucin domain-containing protein 2 (TIM2) and SEMA4D binds CD72. Class 6 semaphorins bind class A plexin receptors and do not require NRPs; for example, SEMA6A binds to plexin A4. SEMA6D carries out different biological activities through plexin A1 depending on its co-receptor (that is, off-track or vascular endothelial growth factor receptor 2 (VEGFR2)). SEMA7A signals are mediated through β1 integrin receptors in both the nervous system and the immune system, and SEMA7A also binds to plexin C1. Plexin C1 is also known as the receptor of viral semaphorins, such as A39R (from poxvirus) and AHV-Sema (from alcelaphine herpesvirus type 1). GAP, GTPase-activating protein.

role in the suppression of antitumour immunity[48]. Third, $CD4^+$ T cell-specific ablation of NRP1 expression results in delayed tumorigenesis in mouse transplanted-tumour models; these tumours contain activated intratumoral $CD8^+$ T cells[7]. Finally, it has recently been reported that NRP1 is important to potentiate T_{Reg} cell functions and survival[49]. In addition, NRP1 has been shown to be crucial for the suppressive activities of T_{Reg} cells in experimental models of antitumour immunity and colitis. Collectively, these findings strongly indicate that NRP1 is involved in T_{Reg} cell-mediated immunosuppression in mice.

A recent report showed that NRP1 expression distinguishes thymus-derived T_{Reg} cells from peripherally derived T_{Reg} cells[8,9]. By comparing gene expression levels between thymus-derived and peripherally derived T_{Reg} cells using microarrays, NRP1 was found to be expressed at high levels by most thymus-derived T_{Reg} cells but not by mucosa-generated peripherally derived T_{Reg} cells. This indicates that distinct types of infiltrating T_{Reg} cells are involved in different inflammatory conditions[8]. In addition, using T cell receptor-transgenic mice that have defined self antigen specificity,

Table 1 | **The roles of semaphorins and their receptors in the immune system**

Semaphorins and their receptors	Expression in immune cells	Binding partners	Activities	Related diseases
NRP1	• T cells • T_{Reg} cells • Tumour cells • Endothelial cells	• Class 3 semaphorins • VEGF • Co-receptor for TGFβ, HGF, PDGF and their receptors • Heparin, integrins, fibronectin and SEMA4A	Inhibitory: T cell activation* and tumour angiogenesis*‡	• Cancer[7] • SLE[117]
Plexin A1	• DCs • Plasmacytoid DCs • Osteoclasts	Class 6 semaphorins	Stimulatory: DC activation*, production of type I interferons*‡ and differentiation of osteoclasts*	• EAE[17] • Osteopetrosis[17]
Plexin A4	• T cells • DCs • Macrophages	Class 6 semaphorins	Inhibitory: T cell activation*	• EAE[69] • Sepsis[70]
Plexin B1	• Microglia • Oligodendrocytes	Class 7 semaphorins	• Stimulatory: microglial activation* and injury of oligodendrocytes* • Inhibitory: differentiation of osteoblasts*	• EAE[79] • HAM[78] • Osteoporosis[10]
Plexin D1	$CD4^+CD8^+$ thymocytes	SEMA3E	Stimulatory: migration of thymocytes into the medulla[91]*	NA
TIM2	• Activated T cells • T_H2 cells	SEMA4A	Stimulatory: T cell activation*	EAE[22]
CD72	• B cells • DCs	SEMA4D	Stimulatory: B cell activation*‡ and DC activation*	SLE[118]
α1β1 integrin	• Monocytes • Macrophages	SEMA7A	Stimulatory: monocyte and macrophage activation*	• EAE[24] • Pulmonary fibrosis[88]
SEMA3A	• T cells • Tumour cells • Endothelial cells	Class A plexins	Stimulatory: differentiation of osteoblasts* Inhibitory: monocyte migration*‡, T cell activation*‡, tumour angiogenesis*‡ and osteoclast differentiation*	• Atopic dermatitis[102] • Allergic rhinitis[104] • Osteoporosis[11] • Rheumatoid arthritis[119] • Multiple sclerosis[120] • SLE[100,101] • Cardiac dysrhythmia[121] • Cancer[122]
SEMA3E	Thymus (especially in the medulla)	Plexin D1	Stimulatory: migration of thymocytes into the medulla[91]*	NA
SEMA4A	• DCs • Activated T cells • T_H1 cells	Class B plexins Plexin D1 TIM2	Stimulatory: T cell activation* and T_H1 cell differentiation*	• EAE or multiple sclerosis[22,96] • Atopic dermatitis[105] • Pigmentary retinopathy[18]
SEMA4B	• T cells • B cells	Not known	Inhibitory: basophil-mediated T_H2 cell skewing[123]*	NA
SEMA4D	• T cells • Activated B cells • DCs	Plexin B1 CD72	Stimulatory: B cell activation*‡, DC activation*‡, microglial activation* and injury of oligodendrocytes*	• EAE[79] • HAM[78] • Immunodeficiency syndrome[21] • Osteopetrosis[10]
SEMA6A	• DCs • Langerhans cells	Not known	Stimulatory: granuloma formation‡	• LC histiocytosis and dermatopathic lymphadenitis[124] • GPA[125]
SEMA6D	• T cells • B cells • NK cells	Plexin A1	Stimulatory: DC activation*	Osteopetrosis[17]
SEMA7A	Activated T cells	• Plexin C1 • α1β1 integrin	Stimulatory: monocyte and macrophage activation*‡	• Contact hypersensitivity[24] • EAE[24] • Pulmonary fibrosis[88]

DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; GPA, granulomatosis with polyangiitis; HAM, HTLV1-associated myelopathy; HGF, hepatocyte growth factor; LC, Langerhans cell; NA, not applicable; NK, natural killer; NRP1, neuropilin 1; PDGF, platelet-derived growth factor; SEMA, semaphorin; SLE, systemic lupus erythematosus; TIM2, T cell immunoglobulin and mucin domain-containing protein 2; TGFβ, transforming growth factor-β; T_H, T helper; T_{Reg}, regulatory T; VEGF, vascular endothelial growth factor. *The activity has been shown in mouse systems. ‡The activity has been shown in human systems.

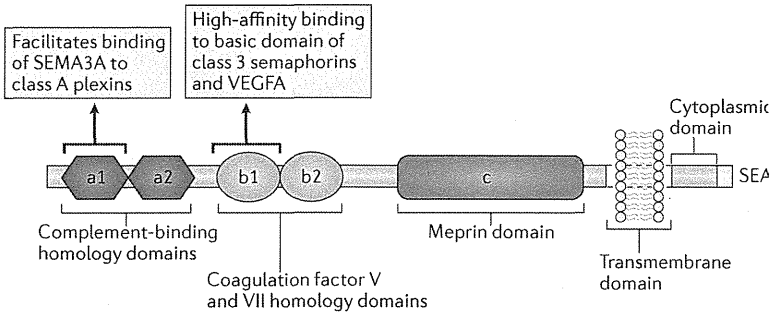

Figure 2 | **The structure and binding sites of neuropilins.** Neuropilins (NRPs) have two complement-binding homology domains (a1 and a2), two coagulation factor V and VII homology domains (b1 and b2) and a meprin domain (c) in their extracellular regions. Cumulative findings indicate that a and b domains are crucial for ligand binding, including binding to semaphorin 3A (SEMA3A) and vascular endothelial growth factor splice variant $VEGF_{165}$. Of note, several studies have shown that the b1 domain mediates the high-affinity binding of NRPs to the basic domain of class 3 semaphorins and to VEGFA[108–111], such that VEGFA and class 3 semaphorins can compete for their binding to the b1 domain of NRPs[111–114]. In addition, it has been suggested that the b1 domain of NRP1 binds with high affinity to the basic domain of SEMA3A, whereas the a1 domain of NRP1 helps the Sema domain of SEMA3A to coordinate with the Sema domain of class A plexins and probably to activate the signalling of class A plexins[114–116]. SEA represent the last amino acid residues (Ser, Glu and Ala) of the cytoplasmic domain, which provide binding to the PDZ (PSD95, DLGA and ZO1 homology) domain-containing protein GIPC1 (also known as synectin).

another study also showed that NRP1 is expressed at high levels in thymus-derived T_{Reg} cells and that it can be used to distinguish between thymus-derived T_{Reg} cells and peripherally derived T_{Reg} cells. This indicates that there are functional differences between these cells[9]. Collectively, these data indicate that NRP1 is a marker that distinguishes thymus-derived from peripherally derived T_{Reg} cells, at least in mice. However, further careful investigation will be required to determine whether NRP1 is a stable marker for thymus-derived T_{Reg} cells, as well as whether these findings are applicable to human T_{Reg} cells. In addition, it still remains unclear what the binding partner for NRP1 on T_{Reg} cells might be.

As noted above, NRP1 functions as a co-receptor for multiple ligands in addition to semaphorins, such as VEGF and TGFβ1, which indicates that semaphorins might interact with or compete with other ligands, thereby altering the signalling outcome; for example, $VEGF_{165}$ promotes microvessel outgrowth, whereas SEMA3A suppresses this effect[50]. As a result of the adhesive properties of the NRP extracellular domains, a considerable number of molecules have been reported to be NRP1 ligands. Although NRP1 is thought to function as a 'hub' receptor for different ligands, such adhesive-binding characteristics can produce controversial and confusing results that require further investigation. Of note, it has recently been reported that SEMA4A binds to NRP1 and is relevant to NRP1-mediated T_{Reg} cell functions and stability[49]. However, there are no apparent defects in the development and functions of $FOXP3^+$ T_{Reg} cells in SEMA4A-deficient mice in physiological conditions[51]. Therefore, regarding the ligands for NRP1 and the mechanisms of NRP1-mediated functions, definitive comprehensive studies using gene-targeted mice and biochemical ligand-binding analysis will be necessary to determine the precise biological roles of NRP1 in T_{Reg} cell-mediated immune functions[35].

Plexins

As NRPs have short cytoplasmic tails and are generally unable to generate signals by themselves, cytoplasmic signalling that is mediated by plexins is considered to be crucial to generate semaphorin-mediated biological functions. Plexins are divided into four classes in vertebrates: class A (plexins A1, A2, A3 and A4), class B (plexins B1, B2 and B3), class C (plexin C1) and class D (plexin D1)[3,5]. The members of the plexin family have highly conserved cytoplasmic domains that encode a GTPase-activating protein (GAP) for RAS-related protein (R-RAS), as well as a GTPase-binding domain and split GAP domains[3,52,53]. In addition, the cytoplasmic domains of plexins associate with other signalling molecules, such as RHO family GTPases, p21-activated kinase (PAK), p190 RHO GAP (also known as RHO GTPase-activating protein 35)[54], PDZ (PSD95, DLGA and ZO1 homology)-RHOGEFs (RHO guanine nucleotide exchange factors)[55,56], flavoprotein monooxygenases (also known as MICALs)[57], the FERM domain-containing GEFs known as FARPs[58,59], RAS-related protein M-RAS[60] and RAP1 (REF. 61). Plexins are crucial for actomyosin contraction and microtubule destabilization, and plexin-mediated signalling has been implicated in the inhibition of integrin-mediated cellular adhesion and cytoskeletal remodelling[4,50,58] (FIG. 3). Furthermore, plexins use tissue- and cell lineage-specific co-receptors, including cytoplasmic and receptor-type protein kinases, which thereby enables semaphorins to carry out diverse functions[17,62,63] (BOX 1). In this section, we focus on several members of the plexin family for which immunological functions have been identified.

Plexin A1 in DC-mediated immune responses. Plexin A1 is a receptor for both class 3 (secreted) and class 6 (transmembrane) semaphorins. SEMA3A binds a receptor complex formed by class A plexins and NRP1 (REF. 33), whereas the class 6 semaphorins SEMA6C and SEMA6D directly bind to class A plexins[25,63]. In the immune system, it has been shown that plexin A1 expression is induced by the MHC class II transactivator (CIITA)[64]. Indeed, plexin A1 is expressed at high levels in mature DCs, but at low or undetectable levels in other immune cells such as macrophages, B cells and T cells. Studies using RNA interference and plexin A1-deficient mice have determined the functional importance of plexin A1 in DC-mediated immune responses. Using short hairpin RNA to target plexin A1 expression, it was shown that plexin A1 is involved in the activation of T cells by DCs[64]. Consistent with those findings, we showed that plexin A1-deficient mice have impaired generation of antigen-specific T cells[17,25]. These studies strongly indicate that plexin A1 is required for DC-mediated T cell responses.

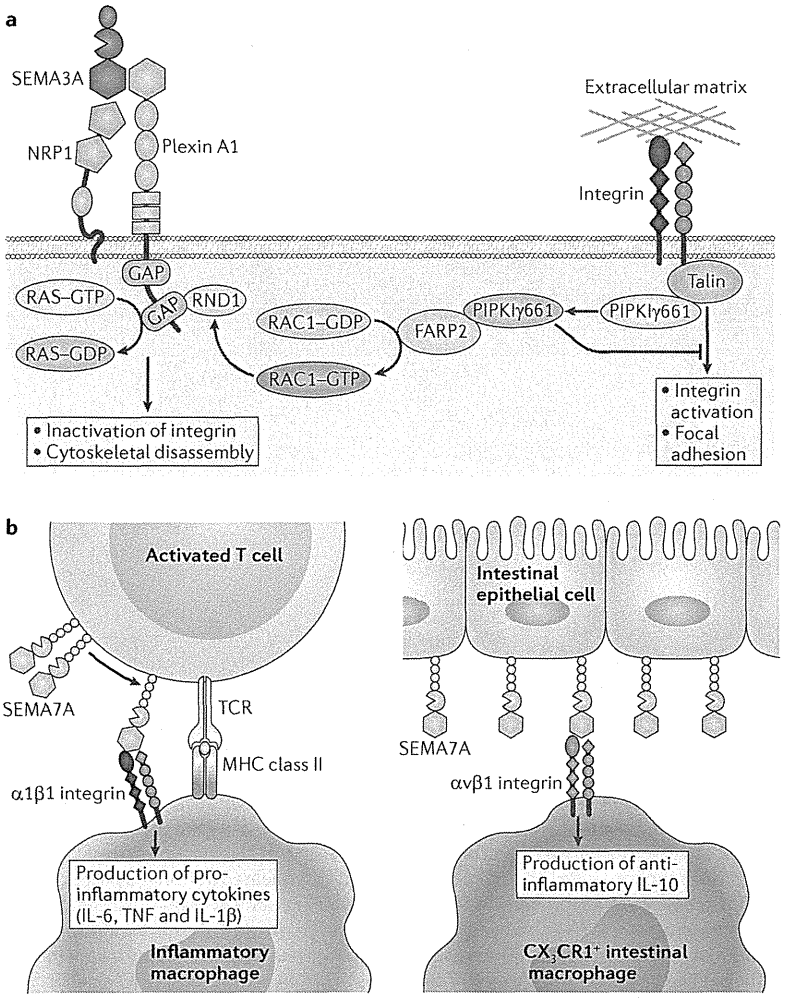

Figure 3 | **Effects of semaphorins on integrin function. a** | Suppression of integrin functions by semaphorin 3A (SEMA3A) is shown. SEMA3A binding to the neuropilin 1 (NRP1)–plexin A1 receptor complex triggers the dissociation of FERM domain-containing GEF 2 (FARP2) from NRP1, which has two major roles. First, the RAC guanine nucleotide exchange factor (GEF) activity of FARP2 is activated, which is essential for subsequent recruitment of RND1 to plexin A1 and for the activation of the RAS-related protein (R-RAS) GTPase-activating protein (GAP) activity of plexin A1, which leads to the downregulation of R-RAS activity. Second, released FARP2 binds to PIPKIγ661 (phosphatidylinositol phosphate kinase type Iγ 661) and inhibits its PIPKIγ kinase activity, which leads to the inhibition of integrin functions and focal adhesion. **b** | SEMA7A positively and negatively regulates immune responses through different integrin receptors. SEMA7A that is expressed on activated T cells stimulates peripheral macrophages through α1β1 integrin, which leads to the production of pro-inflammatory cytokines such as interleukin-6 (IL-6), tumour necrosis factor (TNF) and IL-1β. In addition, SEMA7A that is expressed on intestinal epithelial cells induces IL-10 production by intestinal macrophages through αvβ1 integrin. CX_3CR1, CX_3C-chemokine receptor 1; TCR, T cell receptor.

Regarding the mechanisms of plexin A1 function, our imaging study showed that plexin A1 is involved in sensing SEMA3A during DC migration, particularly for the steps that are involved in transmigration across the lymphatics. During DC migration, plexin A1 is localized at the rear of migrating DCs; in this region, SEMA3A produced by lymphatic endothelial cells induces myosin light-chain phosphorylation to squeeze the cell body, which enables cells to pass through narrow gaps. In addition, adoptive-transfer experiments showed that SEMA3A that is secreted by lymphatic endothelial cells is involved in the regulation of DC trafficking from peripheral tissues to draining lymph nodes. It is plausible that semaphorins (probably class 3 secreted semaphorins) that are produced by vascular endothelial cells[65] are also involved in enabling other immune cells to pass through blood vessel walls by regulating their adhesion activities and contractility in a plexin-dependent manner.

Plexin A1 in osteoimmunology. Osteoimmunology is an interdisciplinary research field, in which the interplay between the skeletal and immune systems is studied at the molecular level. A breakthrough in our understanding of plexin A1-mediated signalling was recently made in the field of osteoimmunology (FIG. 4). Disruption of the gene encoding plexin A1 results in abnormalities in both immune responses and bone homeostasis. Regarding the bone phenotype, plexin A1-deficient mice develop osteopetrosis because of decreased bone resorption that is caused by defective development of osteoclasts; SEMA6D is suggested to function as a ligand for plexin A1 in osteoclast differentiation[17,59]. Indeed, both SEMA6D and plexin A1 are expressed by osteoclasts and recombinant SEMA6D can enhance *in vitro* osteoclastogenesis. Plexin A1 forms a functional receptor complex with triggering receptor expressed on myeloid cells 2 (TREM2) and the adaptor molecule DNAX-activation protein 12 (DAP12; also known as TYROBP) on osteoclasts[17]. However, as both SEMA3A and SEMA6D can use plexin A1 as a receptor component, how do their different modes of action regulate bone homeostasis?

Nrp1-knock-in mice in which the activities of SEMA3A are impaired — through the mutation of the NRP1 a1 domain that is responsible for mediating the interaction between the Sema domains of SEMA3A and class A plexins (FIG. 2) — had osteoporosis that was identical to that of SEMA3A-deficient mice[11]. This study showed that, in the absence of receptor activator of NF-κB ligand (RANKL; also known as TNFSF11), SEMA3A that is produced by osteoblasts binds to NRP1–plexin A1 on osteoclast precursor cells and hinders the interaction between plexin A1 and the TREM2–DAP12 complex, thereby suppressing SEMA6D-induced osteoclastogenesis[11]. By contrast, in the presence of RANKL, the expression of NRP1 is downregulated and SEMA6D binds to the plexin A1–TREM2–DAP12 receptor complex to enhance osteoclastogenesis. The authors also found that SEMA3A repels osteoclast precursor cells to prevent excessive bone disruption, which suggests that the end result of SEMA3A function is decreased osteoclast differentiation and decreased osteopetrosis. In addition to the osteoclast phenotype, deficiency of either SEMA3A or NRP1 results in a decreased number of osteoblasts, decreased expression of osteoblast genes including *Runx2* and alkaline phosphatase liver/bone/kidney isozyme (*Alpl*) and decreased bone formation, which indicates that SEMA3A positively regulates osteoblast differentiation. SEMA3A promotes the activation

Osteopetrosis
A rare inherited disorder characterized by abnormally dense and brittle bones. It is caused by the failure of osteoclasts to resorb bone.

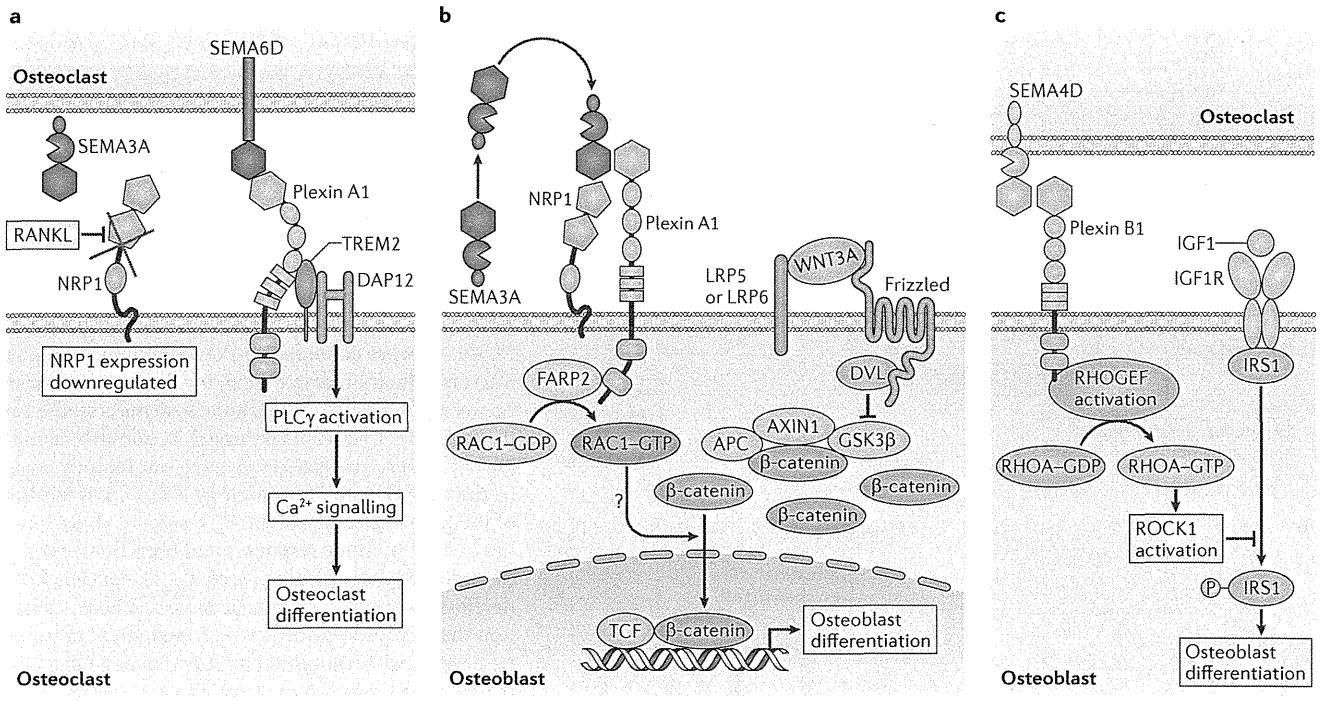

Figure 4 | **Plexin A1- and plexin B1-mediated signalling in bone homeostasis. a** | Semaphorin 6D (SEMA6D)–plexin A1-mediated intracellular signalling in osteoclasts during receptor activator of NF-κB ligand (RANKL) stimulation is shown. In the presence of SEMA6D expressed by osteoclasts, plexin A1 forms a receptor complex with triggering receptor expressed on myeloid cells 2 (TREM2) and DNAX-activation protein 12 (DAP12), which mediates osteoclast differentiation through calcium signalling downstream of phospholipase Cγ (PLCγ) activation. In the presence of RANKL, neuropilin 1 (NRP1) is downregulated in osteoclasts, so SEMA3A does not have an effect on plexin A1 signalling. **b** | The function of the SEMA3A–plexin A1–NRP1 complex in osteoblast differentiation is shown. Soluble SEMA3A, which is released from osteoblasts, binds to plexin A1 and NRP1 on osteoblasts. This complex signals through FERM domain-containing GEF 2 (FARP2) to activate the small G protein RAC1, which subsequently promotes WNT3A-induced accumulation of β-catenin in the nucleus. Thus, this signalling pathway induces the differentiation of osteoblasts. **c** | The crosstalk between plexin B1 and insulin-like growth factor 1 (IGF1) signalling is shown. The binding of SEMA4D to plexin B1 contributes to RHOA activation by RHO guanine nucleotide exchange factors (GEFs) in the intracellular region of plexin B1. RHOA activates the downstream kinase RHO-associated protein kinase 1 (ROCK1), which leads to the suppression of IGF1-mediated signalling through phosphorylation of insulin receptor substrate 1 (IRS1); this therefore leads to the inhibition of osteoblast differentiation. APC, adenomatous polyposis coli; AXIN1, axis inhibitor 1; Dvl, Disheveled; GSK3β, glycogen synthase kinase 3β; IGF1R, IGF1 receptor; LRP, low-density lipoprotein receptor-related protein; TCF, T cell-specific transcription factor.

Osteoclasts
Multinucleated cells of haematopoietic origin that degrade the bone matrix. They have a crucial role in both physiological and pathological bone resorption.

Osteoporosis
A common disease that is characterized by low bone mass, microarchitectural disruption and skeletal fragility, which results in an increased risk of fracture. An oversupply of osteoclasts relative to the need for remodelling or an undersupply of osteoblasts relative to the need for cavity repair are important pathophysiological changes in osteoporosis.

of the small G protein RAC1 through FARP2, which enhances WNT3A-induced nuclear accumulation of β-catenin. β-catenin signalling pathways are essential for the differentiation of mesenchymal precursor cells into osteoblasts or adipocytes in bone homeostasis[11] (FIG. 4b). Therefore, these findings indicate not only that there is crosstalk between semaphorin signalling and WNT signalling but also that targeting semaphorins might be a novel molecular basis for the development of anti-osteoclastogenic agents.

Plexin A4: negative and positive roles in the immune system. In the nervous system, plexin A4 functions as a receptor for SEMA3A and SEMA6A[66]. Plexin A4 has a major role in transducing SEMA3A signalling not only in neurons but also in endothelial cells[67]. However, the roles of plexin A4 in the immune system seem to differ from those of plexin A1. Plexin A4 is expressed by T cells, DCs and macrophages[68], and it has negative regulatory roles in various immune responses[69]. We previously reported that plexin A4-deficient mice have enhanced T cell priming and exacerbated disease in a mouse model of EAE[69]. By contrast, plexin A4 seems to have a positive function in TLR-mediated signalling, as plexin A4 defects in innate immune cells result in decreased inflammatory cytokine production in response to TLR stimuli[70]. It has been suggested that plexin A4 is required for activation of the small GTPase RAC1 and that it thereby modulates JUN N-terminal kinase (JNK) and nuclear factor-κB (NF-κB) activation in macrophages in response to TLR stimuli. Accordingly, plexin A4-deficient mice have attenuated TLR-mediated inflammation, including septic shock[70]. In this situation, SEMA3A — which is upregulated in lymphoid lineage cells such as B cells, T cells, DCs and NK cells following TLR stimulation — functions as a ligand. These findings suggest plexin A4 as a potential therapeutic target for the treatment of sepsis and the related cytokine storm.

Plexin B1 and B2 as receptors for class 4 semaphorins. Class B plexins are most similar to the scatter-factor receptors, which are a family of transmembrane receptors that lead to invasive growth and that are implicated in cancer[71]. Among the class B plexins, the functions of plexin B1 and plexin B2 have been delineated in the context of the class 4 semaphorin SEMA4D[34,72,73]. Plexin B1 has high affinity for SEMA4D[34], which also uses CD72 as an additional receptor in lymphocytes[20,21].

In the immune system, plexin B1 is expressed by activated T cells and immature bone marrow-derived DCs (but not by mature DCs or monocytes)[74], as well as by bone marrow stromal cells, follicular DCs[75], microglia and lung DCs[68,76]. On the basis of its expression pattern, several studies have indicated that through its interactions with SEMA4D, plexin B1 has the following functional roles in the immune system: soluble SEMA4D inhibits the migration of immature DCs and this inhibition can be blocked by plexin B1-specific antibodies[74]; ligation of plexin B1 on B cells by SEMA4D induces increased B cell proliferation and lifespan[75]; plexin B1 expression on renal glomeruli facilitates the recruitment of SEMA4D-expressing macrophages[77]; and SEMA4D activates microglia through plexin B1 and transfer of myelin oligodendrocyte glycoprotein (MOG)-specific T cells into plexin B1-deficient mice results in attenuated development of EAE[78,79].

The signalling pathways downstream of plexin B1 that are triggered by SEMA4D have been delineated in terms of axonal growth-cone collapse — a process in which small GTPases have been implicated as mediators of the biological functions of semaphorins[56,80,81]; for example, plexin B1 activates RHOA through the interaction of the carboxy-terminal PDZ-binding domains of plexin B1 with PDZ-RHOGEF and leukaemia-associated RHOGEF (LARG; also known as RHOGEF12). In addition, SEMA4D induces the recruitment of active RACto the cytoplasmic region of plexin B1, which leads to the inhibition of PAK, which is a downstream effector of RAC[82].

The signalling mechanisms of plexin B1 have also been identified in the field of osteoimmunology (FIG. 4c). Osteoclasts and osteoblasts express SEMA4D and plexin B1, respectively; these interactions inhibit bone formation[10]. During a search for axon-guidance molecules that function in bone remodelling, it was found that the expression of SEMA4D in osteoclasts is upregulated during RANKL-induced osteoclastogenesis, and that deficiency of either SEMA4D or plexin B1 results in high bone mass phenotypes. The PDZ domain of plexin B1 contributes to RHOA activation through RHOGEFs in osteoblasts[10], and dominant-negative RHOA-expressing mice have a high bone mass phenotype similar to that of SEMA4D-deficient and plexin B1-deficient mice. The RHOA–ROCK1 (RHO-associated protein kinase 1) pathway inhibits the phosphorylation of insulin receptor substrate 1 (IRS1), which is involved in IGF1-induced signalling. IGF1 is an important factor for osteoblastogenesis, so its suppression by plexin B1-mediated signals (through the RHOA–ROCK1 pathway) decreases bone formation by osteoblasts. Indeed, SEMA4D suppresses phosphorylation of IRS1 at a tyrosine residue that is essential for AKT and mitogen-activated protein kinase (MAPK) activation, which shows that there is crosstalk between plexin and IGF1 signalling (FIG. 4c).

The functional importance of SEMA4D–plexin B2 interactions has also recently been determined[83,84]. Plexin B2 is involved in the epithelial repair process through its interaction with SEMA4D[84]. THY1$^+$ dendritic epidermal T cells (DETCs) — a type of γδ T cell — express SEMA4D whereas plexin B2 is expressed in keratinocytes; plexin B2 has effects on DETCs and SEMA4D-mediated γδ T cell morphology, which indicates that cytoplasmic signals through SEMA4D can be triggered by plexin B2 as a ligand. SEMA4D-deficient mice have defective DETC responses to keratinocyte damage, which results in delayed healing of cutaneous wounds. In addition, negative regulatory roles of plexin B2 in IL-12 or IL-23 p40 subunit production by DCs have been identified[83].

Plexin C1 and integrins. Both plexin C1 and β1 integrins are receptors for the membrane-associated glycosylphosphatidylinositol (GPI)-anchored semaphorin SEMA7A[24,34,85,86]. Plexin C1 was initially identified as a receptor for SEMA7A and virally encoded semaphorins[34,87]. The viral semaphorins A39R (from poxvirus) and AHV-Sema (from alcelaphine herpesvirus type 1) bind to virus-encoded semaphorin protein receptor (VESPR) (also known as plexin C1 and CD232) and induce the production of pro-inflammatory cytokines, thereby modulating the pathogenesis of these viral infections; these effects can be abrogated with a plexin C1-blocking antibody[87]. Similarly, we have shown that recombinant soluble SEMA7A protein has effects on macrophages that include the upregulation of expression of intercellular adhesion molecule 1 (ICAM1) and the induction of expression of pro-inflammatory cytokines such as tumour necrosis factor (TNF) and IL-6 (REF. 24); this finding substantiates the role of SEMA7A in inflammatory responses.

However, although plexin C1 was initially identified as a receptor for SEMA7A, the biological activities of SEMA7A have been delineated in the context of its interactions with integrins. SEMA7A contains an Arg-Gly-Asp sequence, which is a well-conserved integrin-binding motif. In the immune system, we have shown that SEMA7A that is expressed on activated T cells is involved in inducing the production of pro-inflammatory cytokines by macrophages through α1β1 integrin (also known as VLA1)[24]. Consistent with these findings, SEMA7A-deficient mice are defective in cell-mediated immune responses, including hapten-induced contact hypersensitivity[24] and TGFβ1-induced lung fibrosis[88], in which β1 integrin functions as a receptor component. These findings indicate the importance of the interactions between SEMA7A and α1β1 integrin in T cell-mediated macrophage activation. Moreover, we recently showed that SEMA7A that is expressed on intestinal epithelial cells negatively regulates activation of intestinal macrophages through αvβ1 integrin and that this has an important role in intestinal homeostasis[86]. Thus, SEMA7A has both positive and negative regulatory functions by associating with different types of β1 integrin (FIG. 3).

Scatter-factor receptors
A family of transmembrane receptors, of which MET and RON tyrosine kinases are members. MET is the receptor for hepatocyte growth factor and RON is the receptor for macrophage-stimulating protein.

Plexin D1 in SEMA3E-mediated cellular navigation. Plexin D1 was initially identified because of its key role in development of the vasculature[13,89]; plexin D1 deficiency results in congenital heart defects as a result of improper vessel patterning[89]. SEMA3E and SEMA4A were identified as ligands for plexin D1 (REFS 89,90).

In the immune system, the SEMA3E–plexin D1 axis has a role in thymocyte development[91]. The expression of plexin D1 on thymocytes decreases during development of $CD4^+CD8^+$ double-positive thymocytes to $CD4^+$ or $CD8^+$ single-positive thymocytes, and SEMA3E is preferentially expressed in the medulla of the thymus. Chemotaxis assays carried out *in vitro* have shown that SEMA3E binds to $CD4^+CD8^+CD69^+$ cells and inhibits their CC-chemokine receptor 9 (CCR9)-mediated migration. Consistent with this finding, the thymus in plexin D1-deficient embryos is disorganized compared with the thymus from wild-type control embryos. In addition, when fetal liver cells derived from plexin D1-deficient embryos were transferred to SEMA3E-deficient mice, the boundary between double-positive and single-positive thymocytes at the corticomedullary junction was disrupted[91]. These findings indicate that plexin D1 is involved in the development of thymocytes and of thymic architecture.

Several additional roles for plexin D1 in the immune system have been determined. Plexin D1-mediated signals are relevant to germinal centre formation and long-term B cell immune responses[92]. Plexin D1 is expressed by DCs and its absence results in increased production of the IL-12 and IL-23 p40 subunit[83]. Plexin D1 and its ligand SEMA4A are expressed on macrophages, and macrophage migration towards SEMA4A is abrogated in the presence of plexin D1-blocking antibodies[93]. However, the plexin D1-mediated signalling mechanisms that control these immune responses remain unclear and require further study.

As described here, cumulative findings indicate that semaphorin receptors have diverse roles in several phases of immune responses, from the initiation of a response to the terminal inflammatory immune reactions. Of note, immune cells circulate and interact with other systems such as the nervous, vascular, epithelial and skeletal systems. Therefore, semaphorin receptor-mediated biological activities could enhance our understanding of the 'bigger picture' of physiological and pathological immune responses *in vivo*.

Use as diagnostic and therapeutic targets

In the past few years, it has become clear that semaphorins and their receptors are crucially involved in the pathogenesis of various human diseases and that they are therefore potential diagnostic or therapeutic targets[92,94] (TABLE 1). In the context of the involvement of these proteins in the pathogenesis of immunological disorders, many studies have investigated the relationship between semaphorins and multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus (SLE), allergic diseases and graft-versus-host disease (GVHD). In this section, we focus on the roles of semaphorins in these diseases.

Multiple sclerosis. Multiple sclerosis is a demyelinating autoimmune disease of the central nervous system and a leading cause of lasting neurological disabilities in young adults; EAE is commonly used as an animal model of this disease and has provided evidence of a pathological role for various semaphorins. SEMA3A that is produced in the lymphatics functions as a ligand for the plexin A1–NRP1 receptor complex expressed by DCs, which regulates DC migration from the peripheral tissues to the lymph nodes for antigen presentation to T cells. The lack of SEMA3A–NRP1–plexin A1 interactions results in attenuated development of EAE because of impaired T cell responses[25]. Recent evidence has highlighted the pathological importance of immune cell migration as a therapeutic target in multiple sclerosis; for example, fingolimod (also known as FTY720) and an $\alpha4\beta1$ integrin-specific antibody suppress the relapsing forms of multiple sclerosis by inhibiting cellular migration. Therefore, SEMA3A–NRP1–plexin A1 interactions are potential therapeutic targets for multiple sclerosis.

SEMA4D is highly expressed by T cells and is crucially involved in T cell activation, which requires DC maturation[20,95]. Indeed, SEMA4D-deficient mice show attenuated EAE because of impaired T cell priming[21,95]. It seems that SEMA4D is crucially involved in the pathogenesis of EAE, particularly in the initial phases of pathogenic T cell activation that are mediated by interactions between T cells and DCs.

In addition to its pathological roles in the periphery in terms of T cell activation, we have shown that SEMA4D also contributes to neuro-inflammation in the central nervous system, where SEMA4D that is expressed on the cell surface of T cells induces the activation of microglial cells through plexin B1 as well as inducing the death of immature neural cells. Consistent with these findings, SEMA4D-blocking antibodies inhibited neuro-inflammation, which thereby attenuated the development of EAE[79]. Collectively, these findings indicate that blocking SEMA4D not only inhibits the generation of encephalitogenic T cells but also suppresses the inflammatory neural damage that occurs after clinical onset of EAE. Clinical trials using SEMA4D-blocking antibodies to treat multiple sclerosis have been initiated in the United States (ClinicalTrials.gov, number: NCT01764737).

SEMA4A is highly expressed in DCs and has been shown to have a crucial role in T_H cell differentiation[22,23]. SEMA4A is also involved in the pathogenesis of EAE and multiple sclerosis. Indeed, blocking antibodies that are specific for SEMA4A attenuate EAE, and SEMA4A-deficient mice are resistant to EAE[22,23] as a result of their decreased generation of MOG peptide-specific $CD4^+$ T cells.

We recently reported that the serum levels of soluble SEMA4A are increased in patients with multiple sclerosis[96]. Specifically, in these patients, SEMA4A expression is increased on the cell surface of DCs and SEMA4A is shed from these cells. Patients with high SEMA4A levels have T_H17 cell skewing as well as severe disabilities and unresponsiveness to IFNβ therapy. Taken together, these results not only indicate that SEMA4A is involved

Fingolimod
An oral sphingosine-1-phosphate receptor modulator that sequesters lymphocytes in the lymph nodes, which prevents them from contributing to an immune reaction. It is approved for the treatment of multiple sclerosis, in which it decreases the rate of relapses in relapsing remitting multiple sclerosis.

Table 2 | **Immunological phenotypes of knockout mice of semaphorins and their receptors**

Semaphorins and their receptors	Phenotypes of knockout mice	Refs
SEMA3A	• Impaired T cell priming	25
	• Impaired migration of DCs to the lymph nodes	25
SEMA3E	• Impaired antigen-specific activation of T cells	92
	• Impaired development of thymocytes in the thymus	91
SEMA4A	• Impaired T_H1 cell responses induced by *Propionibacterium acnes*	23
	• Enhanced T_H2 cell responses induced by *Nippostrongylus brasiliensis*	23
	• Impaired antigen presentation by DCs	23
SEMA4B	Enhanced basophil-mediated responses	123
SEMA4D	• Impaired activation of DCs	95
	• Impaired activation of B cells	20
	• Impaired migration of monocytes induced by chemokines	126
	• Impaired secretion of iNOS from microglial cells	79
	• Impaired platelet responses to vascular injury	127
SEMA6D	No defects in T cell priming	25
SEMA7A	• Impaired activation of macrophages	24
	• Resistance to EAE	24
	• Hypersensitivity to EAE	128
	• Resistance to experimental contact dermatitis	24
	• Enhanced responses to DSS-induced colitis	86
	• Resistance to lung fibrosis	88
Plexin A1	Impaired migration of DCs to the lymph nodes	25
Plexin A4	• Enhanced T cell proliferation after TCR stimulation	69
	• Enhanced responses of EAE	69
	• Resistance to septic shock	70
Plexin B1	Impaired SEMA4D-mediated microglial activation	79
Plexin B2	Impaired SEMA4D-mediated microglial activation	79
Plexin C1	Impaired SEMA7A-mediated monocyte activation	129
Plexin D1	Impaired development of thymocytes in the thymus	91

DC, dendritic cell; DSS, dextran sodium sulphate; EAE, experimental autoimmune encephalomyelitis; iNOS, inducible nitric oxide synthase; SEMA, semaphorin; T_H, T helper; TCR, T cell receptor.

in the pathogenesis of multiple sclerosis by promoting T_H17 cell skewing but also suggest that SEMA4A could be a diagnostic or a prognostic marker of multiple sclerosis.

SEMA7A is expressed by activated T cells and interacts with α1β1 integrin that is expressed by macrophages to promote the production of pro-inflammatory cytokines. SEMA7A-deficient mice are resistant to EAE and SEMA7A has been pathologically implicated in the effector phase of EAE through its interaction with α1β1 integrin[24].

Rheumatoid arthritis and SLE. Rheumatoid arthritis is a chronic inflammatory disorder that typically affects small- and medium-sized peripheral joints, in which the articular cartilage and the surrounding bones are destroyed by proliferative synovitis. The synovial lesion in rheumatoid arthritis is formed by inflammatory cell invasion, proliferation of the lining cells and increased angiogenesis, a process in which expression of the $VEGF_{165}$ splice variant and its receptor NRP1 have been implicated[97]. Furthermore, a recent report showed that treatment with an anti-NRP1 peptide could suppress the development of experimental arthritis in mice[98], which indicates that similar peptides could be worth testing for the treatment of chronic arthritis.

$CD4^+$ T cells that are derived from patients with rheumatoid arthritis have defective SEMA3A expression. SEMA3A enhances the suppressive ability of $CD4^+NRP1^+$ T cells, which leads to IL-10 production and regulatory activities[99]. Furthermore, several reports have suggested that SEMA3A is important in the pathogenesis of SLE[100,101]. Serum SEMA3A levels, which are decreased in patients with SLE, inversely correlate with the severity of SLE, including the presence of renal damage and of serum cardiolipin-specific antibodies[100]. These findings indicate that SEMA3A is a potential therapeutic agent for SLE.

Allergic diseases. The pathological implications of semaphorins have also been reported for allergic diseases. The therapeutic effects of SEMA3A on atopic dermatitis have been shown using mouse models in the context of neuro-immune crosstalk. Atopic dermatitis is a chronically relapsing itch or inflammatory skin condition that markedly reduces the quality of life. Itching sensations are conducted by afferent C fibres, which are unmyelinated nerve fibres that originate from neurons of the dorsal root ganglia. In normal conditions, the free nerve endings of C fibres are located at the boundary between the epidermis and the dermis. By contrast, in patients with atopic dermatitis, C fibres in the epidermis increase

Atopic dermatitis
A chronic inflammatory, relapsing and itchy skin disorder. Impaired epidermal barrier functions and allergic responses have important roles in the pathogenesis of atopic dermatitis.

NC/Nga mice
A well-described animal model for atopic dermatitis. In conventional housing conditions, these mice develop skin lesions that are clinically and histologically similar to human atopic dermatitis.

and sprout, probably in response to the nerve growth factor (NGF) that is produced by keratinocytes or fibroblasts in response to scratching, which results in hypersensitivity and more itching[94]. Intracutaneous injection of SEMA3A protein into the skin lesions of NC/Nga mice, which are an animal model of atopic dermatitis, attenuated several symptoms, such as scratching behaviour, erosion and oedema[102]. The validity of this therapeutic strategy is supported by the finding that patients with atopic dermatitis have lower levels of SEMA3A in the epidermis compared with control patients. In addition, it has been reported that the expression of SEMA3A is lower in psoriatic skin than in skin from healthy control patients, whereas the expression of NGF is higher[103]. Given these findings, it is reasonable to conclude that decreased SEMA3A expression is involved in the development of itching and skin inflammation in both atopic dermatitis and psoriasis. On a related note, decreased SEMA3A expression in the nasal mucosa might contribute to nasal hypersensitivity during allergic rhinitis[104], and intranasal administration of recombinant SEMA3A decreases sneezing and nasal rubbing symptoms in mouse rhinitis models[104]. Therefore, it seems that SEMA3A is required for the homeostasis of the C fibres that conduct itching sensations by balancing the effects of NGF.

SEMA4A has been implicated in the regulation of T_H cell differentiation[23], and increased levels of SEMA4A may be involved in the pathogenesis of autoimmunity[96]. By contrast, SEMA4A insufficiency results in allergic diseases, including atopic dermatitis and airway hypersensitivity[105,106]. In a model of ovalbumin-specific experimental asthma, SEMA4A-deficient mice had enhanced airway hyper-reactivity with increased pulmonary eosinophil infiltration, which was associated with increased levels of T_H2-type cytokines and IgE in bronchoalveolar lavage fluid. Consistent with these observations, recombinant SEMA4A protein suppresses T_H2-type cytokine production and the severity of airway hyper-reactivity. Thus, it is plausible that SEMA4A has therapeutic potential for allergic diseases.

GVHD. Acute GVHD is a major complication in allogeneic bone marrow transplantation, in which donor T cells respond to alloantigens on recipient DCs. In mouse allogeneic bone marrow transplantation, it has been shown that mice transplanted with SEMA4D-deficient T cells have decreased mortality and GVHD-mediated target organ damage[107], which shows the potential therapeutic application of blocking SEMA4D in tissue and/or organ transplantation.

As described here, new evidence is emerging that semaphorins and their receptors are crucial for the pathogenesis of several diseases, particularly for diseases in which several biological systems, such as the immune, nervous and vascular systems, are involved. Thus, blocking signalling that is mediated by semaphorins might have beneficial effects not only for attenuating immune responses but also for protecting tissues or promoting tissue repair.

Conclusions

Semaphorins form a family of immunoregulatory molecules. In conjunction with their receptors — mainly neuropilins and plexins — semaphorins mediate multiple biological activities. A lack of semaphorin signalling results in several immune disorders — including autoimmune and allergic diseases — but excess semaphorin signalling can also induce disease. Thus, semaphorins and their receptors have crucial roles in maintaining immunological homeostasis. An increased understanding of the mechanisms by which semaphorins and their receptors regulate the immune system should aid in the development of therapeutic targets for several human diseases.

However, several issues still remain to be clarified. First, although NRPs and plexins have been found to mediate cell motility and morphology through their role as semaphorin receptors in the nervous system, it is unknown how and to what extent they also regulate immune cell trafficking *in vivo*. Second, we need to clarify the molecular basis for the multiple biological activities of semaphorins in different tissues and cells in both physiological and pathological conditions. Third, the details of ligand–receptor interactions remain unclear because of the controversial and confusing nature of findings regarding the adhesive properties of the extracellular domains of NRPs and plexins. Fourth, for the potential clinical application of semaphorins and their receptors, side effects outside of the immune system — for example, in the central nervous and vascular systems — must be considered. To address these issues, careful and definitive evaluation using gene-targeted mice or binding analyses will be crucial (TABLE 2), not only to fully elucidate the functions of these molecules but also to identify potential diagnostic and therapeutic targets for immune disorders.

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