

## ORIGINAL ARTICLE

Human antibodies targeting the C-type lectin-like domain of the tumor endothelial cell marker clec14a regulate angiogenic properties *in vitro*MK Ki<sup>1</sup>, MH Jeoung<sup>1</sup>, JR Choi<sup>1</sup>, S-S Rho<sup>2</sup>, Y-G Kwon<sup>2</sup>, H Shim<sup>3</sup>, J Chung<sup>4</sup>, HJ Hong<sup>5</sup>, BD Song<sup>1</sup> and S Lee<sup>1</sup>

It has been suggested that clec14a may be involved in tumor angiogenesis. However, a molecular mechanism has not been clearly identified. In this study, we show for the first time that C-type lectin-like domain (CTLCD) of clec14a may be important for regulating cell migration and filopodia formation. Using phage display technology, recombinant human antibodies specific to the CTLCDs of human and mouse clec14a (clec14a-CTLCD (immunoglobulin G) IgG) were selected. Functional assays using the antibodies showed that clec14a-CTLCD IgGs specifically blocked endothelial cell migration and tube formation without affecting cell viability or activation. Further, clec14a-CTLCD IgGs inhibited clec14a-mediated cell–cell contact by blocking interaction between CTLCDs. Finally, clec14a cross-linking by the clec14a-CTLCD IgGs significantly downregulated clec14a expression on the surface of endothelial cells. These results strongly suggest that the clec14a-CTLCD may be a key domain in angiogenesis, and that clec14a-CTLCD IgGs specifically inhibit angiogenesis by modulating CTLCD-mediated cell interactions and clec14a expression on the surface of endothelial cells.

*Oncogene* (2013) 32, 5449–5457; doi:10.1038/onc.2013.156; published online 6 May 2013

**Keywords:** Clec14a; C-type lectin-like domain; antibody; angiogenesis; endothelial cell; regulation

## INTRODUCTION

The rapid recent development of recombinant antibody technology has produced ~30 antibodies approved for human therapy, with more than 270 currently in clinical development for a range of diseases.<sup>1,2</sup> However, conventional antibody selection methods remain time- and labor-intensive and expensive. Traditionally, target protein extracellular regions were used to select antibodies. Consequently, most selected antibodies bind to cells, but are not functional antibodies with therapeutic potential. Owing to recent advances in molecular biology and protein biochemistry, large amounts of information on protein domains and motifs that could link these structures to cell functions are available. Use of a functional domain to select recombinant antibodies may be an effective means of identifying functional antibodies and investigating underlying modes of action.

Tumor angiogenesis has an important role in tumor progression. Vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) are key factors in angiogenesis,<sup>3,4</sup> and targeting angiogenesis is a promising strategy for cancer treatment. The anti-VEGF antibody bevacizumab is used to treat patients with metastatic colorectal cancer, renal cell carcinoma, non-small-cell lung cancer and malignant brain glioma.<sup>5,6</sup> Cetuximab, an anti-EGFR antibody, may inhibit endothelial cell–cell contact and expression of angiogenic factors, such as VEGF, interleukin-8 and basic fibroblast growth factor (bFGF).<sup>7–10</sup> However, because of redundancy of tumor-secreted angiogenic factors, including placental growth factor, angiopoietin, bFGF and

hepatocyte growth factor, these drugs generate a resistant phenotype in tumors.<sup>11,12</sup> Identification of new therapeutic targets may provide viable alternatives for treating cancer patients by inhibition of angiogenesis.

Clec14a is a type I transmembrane protein whose extracellular domain consists of a C-type lectin-like domain (CTLCD), a series of epidermal growth factor-like domains, and a sushi-like domain. Rho *et al.*<sup>13</sup> reported that clec14a is endothelial cell-specific and may have a key role in cell–cell contact in angiogenesis. Mura *et al.*<sup>14</sup> showed that clec14a is critical for regulating pro-angiogenic phenotypes associated with filopodia formation, cell migration and endothelial tube formation, and identified clec14a as a tumor endothelial cell marker not expressed on the endothelium of normal tissues.

In this study, we identified CTLCD functions in cell migration and filopodia formation, key events of angiogenesis. Using phage display technology, we selected recombinant human antibodies against human and mouse clec14a-CTLCDs. Functional assays showed that the antibodies specifically inhibited endothelial cell migration and tube formation without affecting viability or activation. We proposed a mechanism of action whereby the antibodies may inhibit angiogenesis by modulating CTLCD-mediated cell–cell interaction and downregulating clec14a expression on the surface of endothelial cells. In summary, this study uses human clec14a-CTLCD immunoglobulin G (IgGs) to validate CTLCD functions in endothelial cells and provides the first insights into the potential of CTLCD as a therapeutic angiogenic target during cancer progression.

<sup>1</sup>Laboratory of Molecular Cancer Therapeutics, Scripps Korea Antibody Institute, Chuncheon, Korea; <sup>2</sup>Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul, Korea; <sup>3</sup>Division of Life and Pharmaceutical Sciences, Ewha Womans University, Seoul, Korea; <sup>4</sup>Department of Biochemistry and Molecular Biology, College of Medicine, Seoul National University, Seoul, Korea and <sup>5</sup>Department of Systems Immunology, College of Biomedical Science, Kangwon National University, Chuncheon, Korea. Correspondence: Dr S Lee, Laboratory of Molecular Cancer Therapeutics, Scripps Korea Antibody Institute, Hyoja-2-dong, Chuncheon 200-701, Korea.

E-mail: Lees@skai.or.kr

Received 21 September 2012; revised 14 March 2013; accepted 18 March 2013; published online 6 May 2013

**RESULTS**

Clec14a-CTL D may have a key role in cell migration and filopodia formation

To elucidate the role of CTL D in clec14a-mediated cell migration, we transfected COS-7 cells with green fluorescent protein (GFP), wild-type clec14a fused to GFP (clec14a-GFP) or a clec14a-CTL D deletion mutant fused to GFP (clec14a $\Delta$ CTL D-GFP) (Figure 1a). After confirming expression of the transfected plasmids with immunoblot analysis (Figure 1b), we assayed migration in a wound healing assay 0 and 20 h. after transfection. The extent of migration of cells expressing clec14a-GFP was ~1.6-fold greater than that of cells expressing GFP alone. Cells expressing clec14a $\Delta$ CTL D-GFP displayed only minor changes in migration (Figures 1c and d).

To determine the role of CTL D in filopodia formation in endothelial cells, we transfected human umbilical vein endothelial cells (HUVECs) with GFP, clec14a-GFP, or clec14a $\Delta$ CTL D-GFP and performed immunocytochemistry. Clec14a-GFP significantly increased filopodia formation, whereas GFP and clec14a $\Delta$ CTL D-GFP had minimal effect, suggesting that clec14a-CTL D may contribute to filopodia formation in HUVECs (Figures 2a-c). We obtained similar results with COS-7 cells overexpressing GFP, clec14a-GFP or clec14a $\Delta$ CTL D-GFP, supporting the notion that clec14a-CTL D is critical for promoting filopodia formation (data not shown).

These results suggest that CTL D may have a crucial role in endothelial cell migration and filopodia formation.

Isolation of CTL D-specific single-chain variable fragments

A human synthetic single-chain variable fragments (scFv) library was precleared to remove Fc binders; removal of ~90% was confirmed by phage enzyme-linked immunosorbent assay (ELISA; data not shown). The library was alternately biopanned with human (hCTL D-Fc) or mouse (mCTL D-Fc) CTL D fusion proteins,

using CTL D-Fc-coated immunotubes and magnetic beads, to isolate clones with cross-species CTL D reactivity (Figure 3a); several were identified only with beads (Figures 3b-d). Ninety-six phage clones were randomly selected, rescued by helper phage infection, and tested for reactivity to human and mouse CTL Ds in phage enzyme immunoassay. Clone DNA was sequenced and four clones,<sup>1-4</sup> recognizing both human and mouse CTL Ds, and with different complementarity-determining-region sequences, were selected (Supplementary Figure 1).

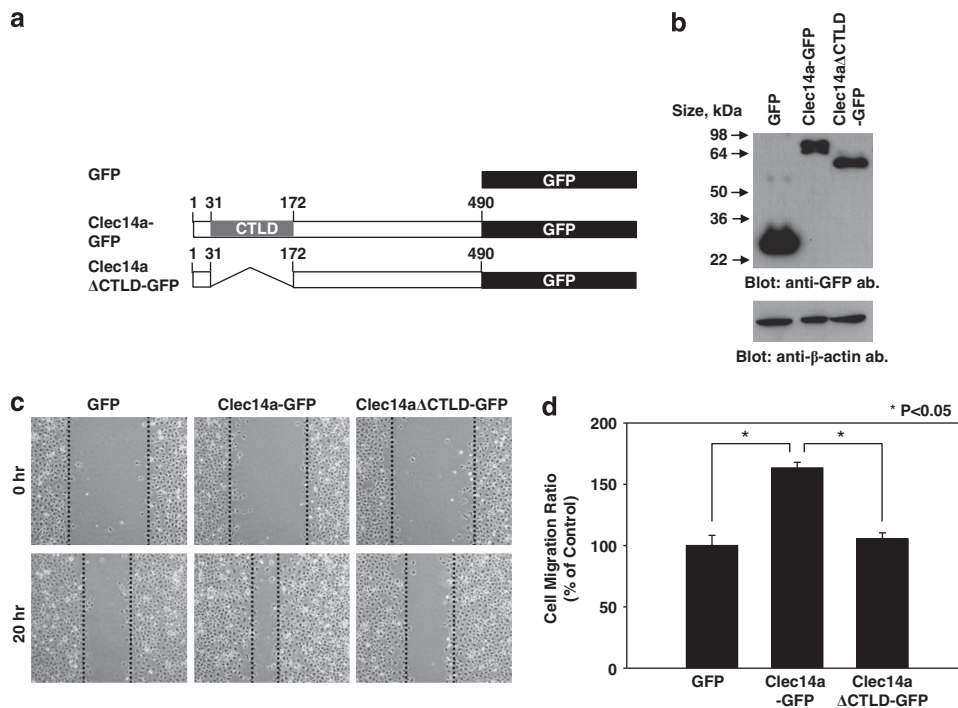
Clec14a-CTL D IgGs specifically recognized human and mouse clec14a-CTL Ds

The scFv clones were converted to IgG, expressed in human embryonic kidney (HEK) 293F cells and purified. The four IgG clones were shown to be >90% pure by SDS-polyacrylamide gel electrophoresis and Coomassie staining (data not shown). ELISA showed that the purified CTL D-specific IgGs (clec14a-CTL D IgGs) specifically bound both human and mouse CTL D-Fc, and not Fc alone. Clones 1 and 2 showed much greater affinity than 3 and 4 (Figure 4).

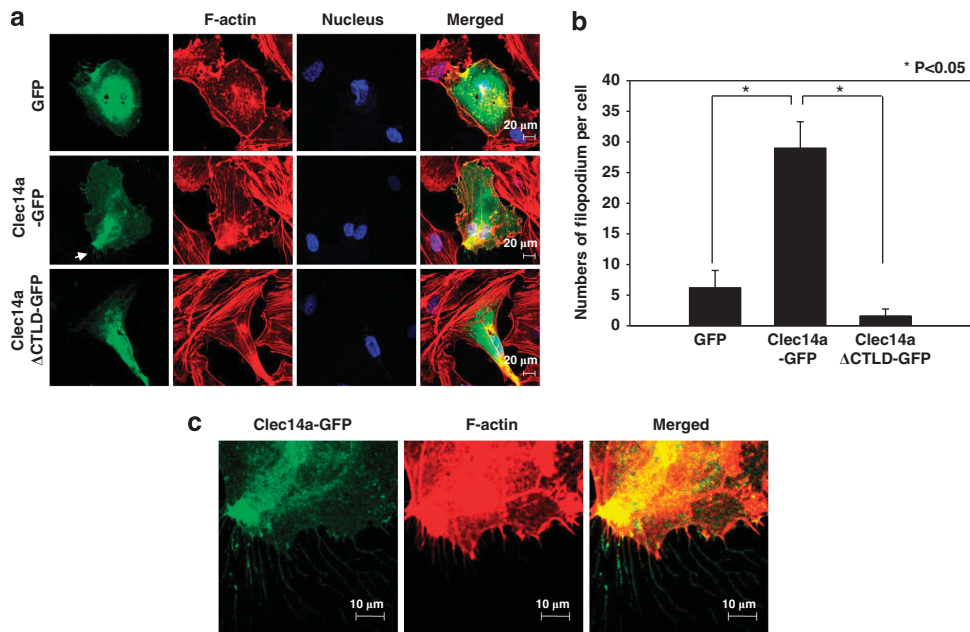
Clec14a-CTL D IgGs specifically suppressed HUVEC cell migration and tube formation

To investigate the inhibitory effect of clec14a-CTL D IgGs on endothelial cell migration, we performed wound healing assays with HUVECs in the absence or presence of clec14a-CTL D IgGs. Cetuximab, an anti-EGFR antibody, was used as control IgG. Of the selected clec14a-CTL D IgGs, clones 1 and 2 significantly suppressed HUVEC cell migration to ~44% and 54%, respectively, whereas clones 3 and 4, and cetuximab alone had little effect (Figures 5a and b).

To determine the effect of clec14a-CTL D IgGs on tube formation, tube formation was assayed in the absence or presence of the clec14a-CTL D IgGs and cetuximab. Clones 1 and 2, and



**Figure 1.** Effect of clec14a-CTL D in cell migration. (a) Schematic representation of GFP, clec14a-GFP and clec14a $\Delta$ CTL D-GFP. (b) Lysates of COS-7 cells transfected with GFP, clec14a-GFP or clec14a $\Delta$ CTL D-GFP were analyzed by immunoblotting with anti-GFP or anti- $\beta$ -actin antibodies. (c) Migration of COS-7 cells transfected with GFP, clec14a-GFP or clec14a $\Delta$ CTL D-GFP in the wound healing assay was monitored under a light microscope. Images were captured at 0 h (top) and 20 h (bottom). (d) Distance migrated is expressed as percent of control migration. Values represent mean  $\pm$  s.d. of triplicate measurements from one of three independent experiments.



**Figure 2.** Effect of clec14a-CTLD on filopodia formation. **(a)** HUVECs transfected with GFP, clec14a-GFP or clec14a $\Delta$ CTL-D-GFP were fixed, stained with rhodamine-phalloidin and Hoechst, and examined by confocal microscopy ( $\times 600$ ). **(b)** The numbers of filopodia per cell were counted and expressed in a bar graph. **(c)** The magnified images ( $\times 2.5$ ) represent the regions of filopodia formation and are indicated by arrows in panel **a**. Results are representative of three independent experiments.

cetuximab<sup>10</sup> specifically blocked HUVEC tube formation, whereas 3 and 4 did not have a significant effect (Figures 5c and d), suggesting that clec14a-CTLD IgGs specifically inhibit endothelial cell migration and tube formation.

#### Effect of Clec14a-CTLD IgGs on endothelial cell viability and activation

To investigate the effect of clec14a-CTLD IgGs on endothelial cell viability, HUVECs were cultured in the absence or presence of clec14a-CTLD IgGs, cetuximab or 5-fluorouracil (5-FU), an apoptosis inducer, for 2 days, and cell viability was checked using a cell counting kit. The antibodies had little effect on HUVEC viability, whereas 5-FU specifically decreased viability (Figure 6a).

To exclude the possibility that the antibodies were toxic, HUVECs were cultured in the absence or presence of clec14a-CTLD IgGs (clone 1), stained with rhodamine-phalloidin, and the cell morphology and F-actin structures were observed using confocal microscopy. No morphological changes to indicate cell death in the presence of the antibodies were observed (Figure 6b).

To further confirm that the effect of clec14a-CTLD IgGs on clec14a-mediated cell viability was not due to antibody toxicity, COS-7 cells overexpressing GFP or clec14a-GFP were cultured in the absence or presence of clec14a-CTLD IgGs (clone 1) for 2 days and cell viability was measured. No changes in the cell viability of COS-7 cells overexpressing GFP or clec14a-GFP were observed in the presence of antibodies (Figure 6c).

To determine the effect of clec14a-CTLD IgGs on endothelial cell activation, HUVECs were cultured in the absence or presence of clec14a-CTLD IgGs, cetuximab, or human tumor necrosis factor alpha (hTNF $\alpha$ ). Activation was determined based on the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular CAM-1 (ICAM-1) measured by flow cytometry. Cells were treated with hTNF $\alpha$  that induces upregulation of VCAM-1 and ICAM-1, as a positive control. The antibodies and cetuximab had no effect on HUVEC activation (Figure 6d), suggesting that clec14a-CTLD IgGs have little effect on endothelial cell viability or activation.

Clec14a-CTLD IgGs specifically inhibited clec14a-mediated cell-cell contact

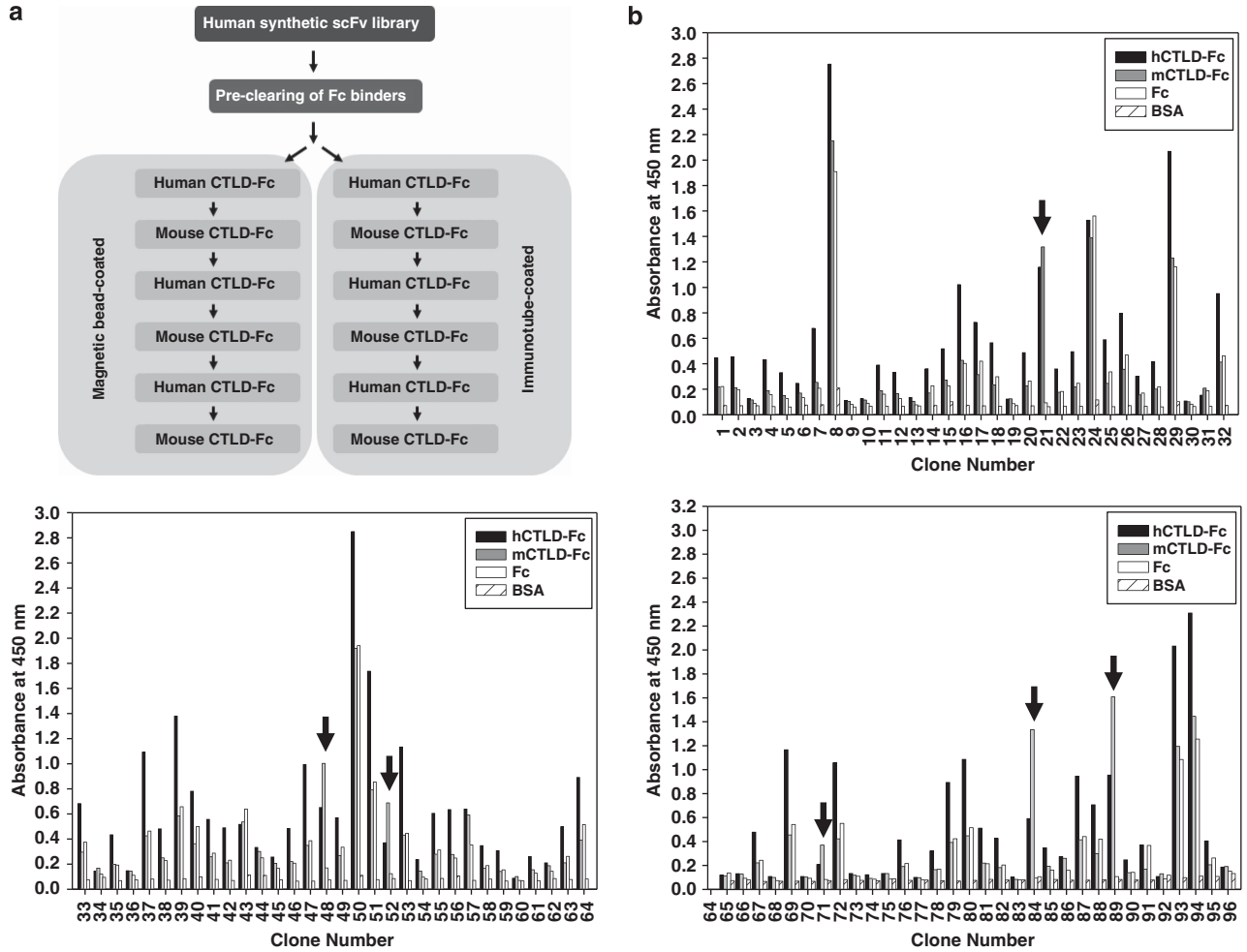
A recent study suggested that clec14a-CTLD is important for endothelial cell-cell contact.<sup>13</sup> To elucidate whether clec14a-CTLD IgGs have an inhibitory effect on endothelial cell-cell contact, the number of cell aggregates, an indicator of clec14a-mediated cell-cell contacts,<sup>13</sup> formed by HEK293F cells transfected with GFP or clec14a-GFP, and grown in the absence or presence of clec14a-CTLD IgGs or cetuximab was determined. The number of cell aggregates was  $\sim$ fourfold greater in cells expressing clec14a-GFP than in those expressing GFP alone (Figures 7a and b). Furthermore, clones 1 and 2 substantially suppressed the aggregation of cells transfected with clec14a-GFP, whereas the other clones and cetuximab had little effect.

To exclude the possibility of antibody toxicity in this assay, we measured the cell viability of HEK293F cells transfected with GFP or clec14a-GFP at the same experimental settings detailed in Figures 7a and b. 5-FU was used as a positive control for cell death. As shown in Figure 7c, clec14a-CTLD IgG had little cytotoxicity in cell-cell contact assays.

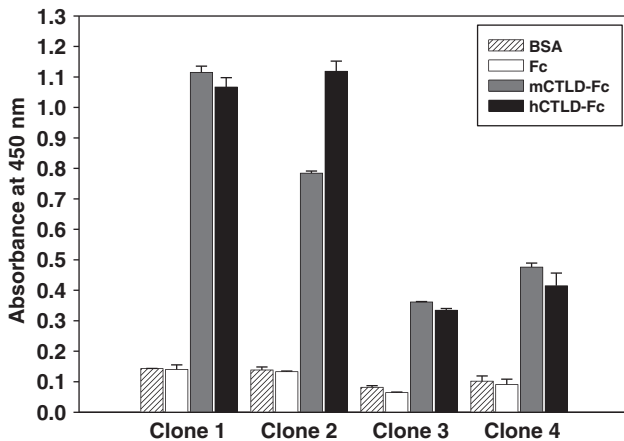
To further confirm that clec14a-CTLD IgGs specifically inhibited clec14a-mediated endothelial cell-cell contact, we performed a cell ELISA in the presence or absence of clec14a-CTLD IgGs (clone 1) on HUVECs that had been incubated with hCTL-D-Fc. clec14a-CTLD IgGs specifically inhibited hCTL-D-Fc binding to HUVECs in a concentration-dependent manner. We also noted that Fc alone was virtually unable to bind HUVECs (data not shown). These results suggest that clec14a-CTLD IgGs have an inhibitory role in clec14a-mediated endothelial cell-cell contact.

Clec14a-CTLD IgG specifically blocked clec14a-CTL-D-CTL-D interactions

To better understand the interaction between clec14a-CTL-Ds, we performed ELISAs on COS-7 cells transfected with GFP, clec14a-GFP or clec14a $\Delta$ CTL-D-GFP. Lysates were incubated with hCTL-D-Fc or Fc to observe interactions between CTL-Ds. Although hCTL-D-Fc may interact with other proteins in COS-7 cell lysates, it



**Figure 3.** Isolation of scFv clones specific to human and mouse CTLDs. (a) A human synthetic scFv antibody library was precleared of Fc binders and screened by alternative biopanning with recombinant hCTL D-Fc or mCTL D-Fc. (b) Ninety-six phage clones (1–96) displaying scFv were randomly selected and the supernatants were analyzed by phage ELISA. Reactivity of the selected scFv clones to human and mouse CTLDs was assayed by measuring absorbance at 450 nm. Arrows indicate scFv clones reactive to hCTL D-Fc (■) and mCTL D-Fc (▒), but not Fc (□). BSA (▨) served as a background control.



**Figure 4.** Cross-species reactivity of clec14a-CTL D IgGs to human and mouse CTLDs. ELISA was performed with purified, selected IgG scFv clones (clones 1–4) on 96-well microtiter plates coated with hCTL D-Fc (■), mCTL D-Fc (▒) and Fc (□). BSA (▨) served as a background control. Values represent mean  $\pm$  s.d. of triplicate measurements from one of two independent experiments.

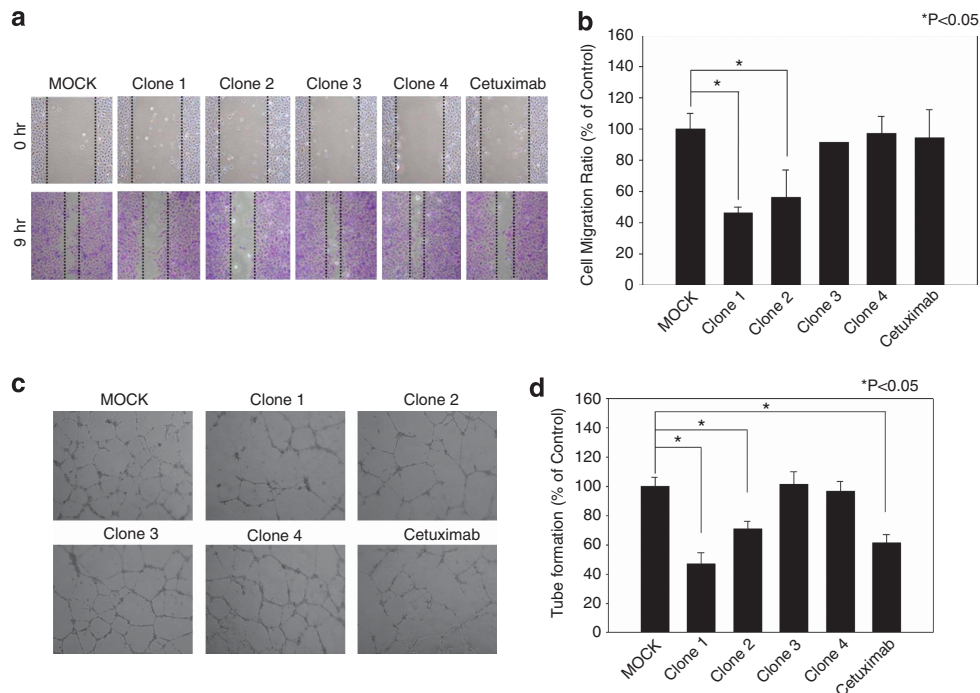
bound strongly to clec14a-GFP but not to clec14a $\Delta$ CTL D-GFP, suggesting a specific CTL D–CTL D interaction in endothelial cells (Figure 8a).

To determine if clec14a-CTL D IgG blocks CTL D–CTL D interactions, hCTL D-Fc was incubated with increasing concentrations of clec14a-CTL D IgG (clone 1), and the protein complexes were incubated with lysates of GFP- or clec14a-GFP-transfected cells. Competitive ELISA showed that clec14a-CTL D IgG specifically inhibited CTL D–CTL D interactions in a concentration-dependent manner (Figure 8b), suggesting that clec14a-CTL D IgG may specifically block clec14a-CTL D–CTL D interactions in endothelial cells.

Cross-linking with clec14a-CTL D IgG downregulated clec14a on the surface of HUVECs

To investigate a possible role of clec14a-CTL D IgG on down-regulating the expression level of clec14a on the surface of HUVECs, we used flow cytometry to compare the level of clec14a expression on HUVEC membranes before and after treatment with clec14a-CTL D IgG (clone 1). IgG treatment significantly reduced clec14a on live, but not paraformaldehyde-fixed, cells (Figure 9a).





**Figure 5.** Effect of clec14a-CTLG on endothelial cell migration and tube formation. **(a)** After wounding, migration of HUVECs incubated in the absence (MOCK) or presence of clec14a-CTLG (clones 1–4) or cetuximab was monitored by light microscopy. Images were captured at 0 h (top) and 9 h (bottom). **(b)** Distance migrated is expressed as percent of control (MOCK) migration. Values represent mean  $\pm$  s.d. of triplicate measurements from one of three independent experiments. **(c)** Tube formation was assayed in the absence (MOCK) or presence of clec14a-CTLG (clones 1–4) or cetuximab. **(d)** Extent of tube formation is expressed as percent of control (MOCK) tube formation. Values represent mean  $\pm$  s.d. of triplicate measurements from one of three independent experiments.

To confirm downregulation of clec14a on HUVEC membranes by clec14a-CTLG, cells were treated with IgG (clone 1) or Fc, and membrane clec14a was measured by cell ELISA. Clec14a-CTLG IgG downregulated clec14a on HUVEC membranes in a time-dependent manner (Figure 9b). These results strongly suggest that clec14a-CTLG cross-linking of clec14a specifically downregulates clec14a on endothelial cell membranes.

## DISCUSSION

Angiogenesis is a hallmark of cancer. Increasing attention is paid to the mechanisms by which tumors promote new blood vessel formation,<sup>3,15</sup> and angiogenesis is a promising target for cancer treatment.<sup>5,6</sup> However, an angiogenic phenotype is complex and results from the coordinated action of many pro- and anti-angiogenic molecules.<sup>4</sup> In this study, we developed recombinant human clec14a-CTLG-specific IgGs and evaluated their efficacy and mechanism of action. We propose that clec14a-CTLG may be a key factor in angiogenesis, and clec14a-CTLG-specific antibodies may have therapeutic potential.

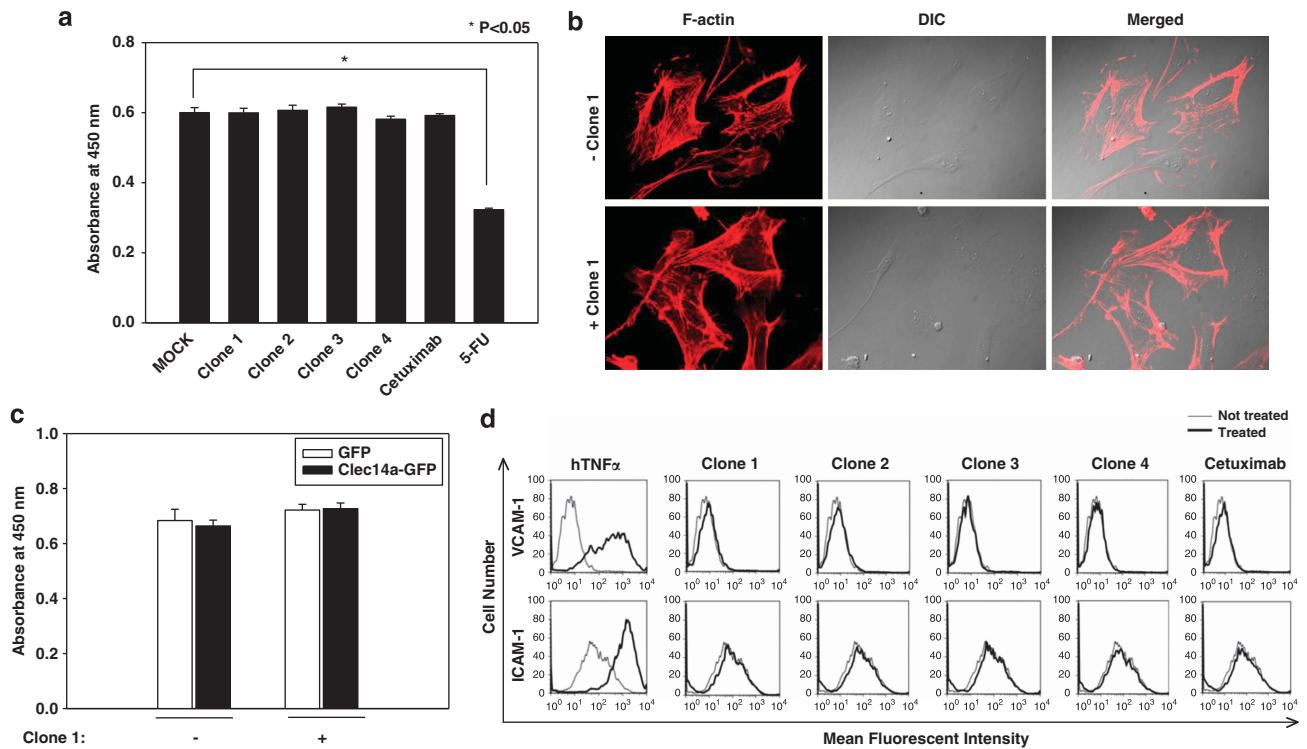
Several reports have suggested that clec14a has a key role in angiogenesis.<sup>13,14</sup> However, the molecular mechanisms underlying this function have not been clearly identified. We suggest that clec14a-CTLG may be a unique domain that regulates angiogenesis in a CTLG–CTLG interaction-dependent manner. First, clec14a-CTLG, particularly amino acids 31–172, had a key role in clec14a-mediated cell migration by regulating filopodia dynamics. Second, consistent with our observation that clec14a-CTLG IgG specifically suppresses clec14a-mediated cell–cell contacts, Rho *et al.*<sup>13</sup> demonstrated that CTLG is important for endothelial cell–cell contact. Third, clec14a modulation by clec14a-CTLG IgG specifically inhibited HUVEC migration and tube formation. Finally, formation of clec14a-CTLG–CTLG complexes was specifically inhibited by clec14a-CTLG IgG. In this study,

we used GFP fusion proteins to elucidate the specific roles of clec14a-CTLG on angiogenic functions *in vitro* and thus cannot exclude the possibility that the fusion proteins may not have identical activities to those of wild-type clec14a.

The human VEGF antibody bevacizumab is currently used to treat patients with a variety of cancers.<sup>5,6</sup> However, because the VEGF receptor is also expressed on normal cells, its use is likely to be associated with adverse effects, including hypertension, proteinuria and gastrointestinal perforation.<sup>16–18</sup> Adverse effects may also limit the therapeutic use of many antibodies against pro-angiogenic factors, such as VEGF receptor-2 and angiopoietin-2.<sup>19–21</sup> Consequently, identification of cancer-specific targets is critical for developing therapeutic antibodies with fewer adverse effects.

The human antibodies to clec14a-CTLG we developed in this study recognize human and mouse CTLG. Broad cross-species reactivity is critical for use in preclinical studies to gain an understanding of antibody function and mode of action prior to clinical trials. Although real-time interaction analysis with BIA-CORE 2000 indicated that the  $K_D$  constant for the interaction between clec14a-CTLG IgG (clone 1 and clone 2) and hCTLG-Fc was  $\sim 1.7 \times 10^{-7}$  and  $6.1 \times 10^{-7}$ , respectively (data not shown), the clec14a-CTLG IgGs specifically inhibited endothelial cell migration and tube formation without affecting cell viability and activation. Recently, others reported that clec14a is expressed exclusively on endothelial cells<sup>13</sup> and may be a specific tumor endothelial cell marker.<sup>14</sup> Although further optimization such as affinity maturation is needed, it is reasonable to speculate that the clec14a-CTLG antibodies might have fewer adverse effects in normal endothelium, target clec14a expressed exclusively on tumor endothelium and suppress angiogenesis during clec14a-mediated tumor progression.

Bevacizumab is a therapeutic antibody<sup>9,11,18</sup> that suppresses angiogenesis by inhibiting interaction between soluble VEGF and its receptors. However, long-term use of bevacizumab generates a



**Figure 6.** Effect of clec14a-CTLG on endothelial cell proliferation and activation. **(a)** HUVECs were incubated in the absence (MOCK) or presence of clec14a-CTLG, cetuximab or 5-FU (positive control) for 2 days. Cell viability was assessed by measuring absorbance at 450 nm. Values represent mean  $\pm$  s.d. of triplicate measurements from one of two independent experiments. **(b)** HUVECs cultured in the absence or presence of clec14a-CTLG (clone 1) were stained with rhodamine-phalloidin and examined by confocal microscopy. **(c)** COS-7 cells transfected with GFP or clec14a-GFP were incubated in the absence or presence of clec14a-CTLG (clone 1). Cell viability was assessed by measuring absorbance at 450 nm. Values represent the mean  $\pm$  s.d. of triplicate measurements from one of two independent experiments. **(d)** HUVECs were cultured in the absence (dashed line) or presence (solid line) of hTNF $\alpha$ , clec14a-CTLG or cetuximab; stained with anti-VCAM-1 (upper) or ICAM-1 (lower) polyclonal antibody and analyzed by flow cytometry. hTNF $\alpha$  served as a positive control for endothelial cell activation. Results are representative of three independent experiments.

resistant tumor phenotype due to redundancy of tumor cell-secreted pro-angiogenic growth factors.<sup>11,12</sup> This may pose the greatest challenge to using antibodies against soluble growth factors in patients requiring long-term therapy. Clec14a is a type I transmembrane protein critical for endothelial cell-cell contact<sup>13</sup> with a different mechanism of action than bevacizumab. The clec14a-CTLG IgG developed in this study appears to have dual mechanisms of action to suppress angiogenic properties *in vitro*. It specifically blocked CTLD-CTLG interaction in a concentration-dependent manner and clec14a cross-linking by IgG induced clec14a downregulation on endothelial cell membranes. Within 2 h of cross-linking, clec14a-CTLG IgG appeared in a dot-like pattern inside HUVECs (data not shown), suggesting antibody-induced endocytosis. Although this study focused on identifying the mechanisms underlying clec14a-CTLG IgG as a blockade of clec14a in angiogenesis, we cannot exclude the possibility that endogenous clec14a ligands exist and other CTLD-binding partners may be involved in clec14a-mediated angiogenesis.

On the basis of the currently available evidence, we propose a mode of action for clec14a-CTLG in endothelial cells during angiogenesis. The clec14a-CTLG domain is critical for regulation of filopodia dynamics in migrating endothelial cells and contributes to endothelial cell-cell contact via CTLD-CTLG interaction. Furthermore, clec14a-CTLG IgGs specifically inhibit pro-angiogenic activities such as endothelial cell migration, cell-cell contact and tube formation by simultaneously blocking CTLD-CTLG interaction and inducing clec14a downregulation on the surface of endothelial cells. Although the current study is based on *in vitro* results, in near future we plan to test this

hypothesis by examining the *in vivo* functional relevance of clec14a-CTLG using optimized clec14a-CTLG IgGs.

## MATERIALS AND METHODS

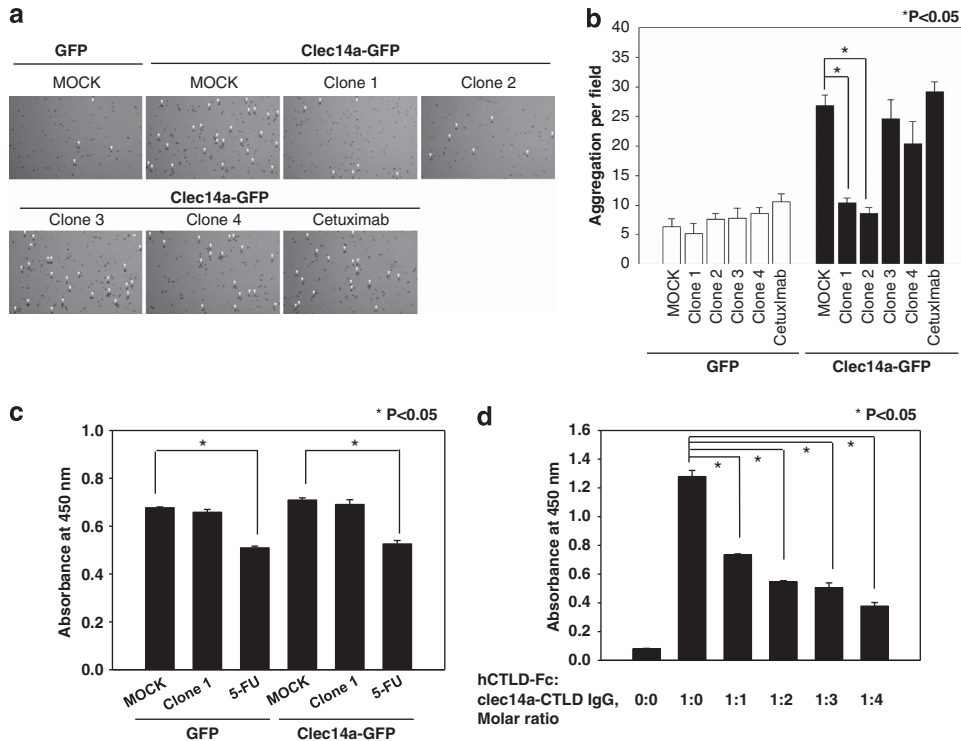
### Cell culture and transfection

HUVECs (Lonza, Walkersville, MD, USA) were maintained in endothelial growth medium-2. COS-7 cells were grown in Dulbecco's modified Eagle medium containing 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. Cells were maintained in a humidified, CO<sub>2</sub>-controlled incubator (Sanyo, Panasonic Healthcare Company, Secaucus, NJ, USA) at 37 °C and 5% CO<sub>2</sub>. HEK293F cells were maintained in Freestyle 293 expression media (Invitrogen, Carlsbad, CA, USA) supplemented with 1% (v/v) penicillin/streptomycin in a humidified Multitron incubation shaker (Infors HT, Bottmingen, Switzerland) at 37 °C and 8% CO<sub>2</sub>. HUVECs and COS-7 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

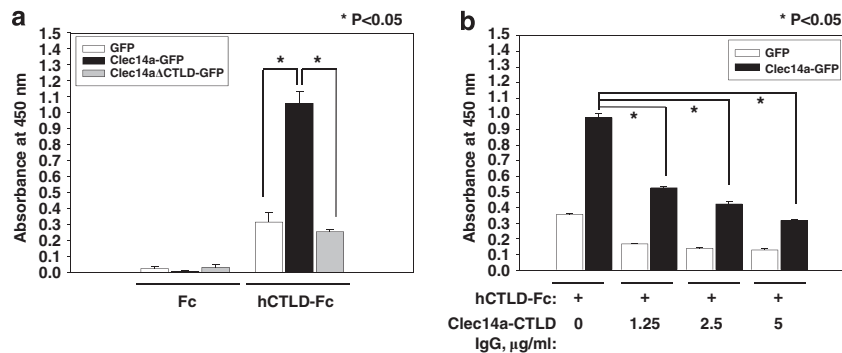
### Wound healing and cell migration assay

A CytoSelect 24-well wound healing assay kit (Cell Biolabs Inc., San Diego, CA, USA) was used according to the manufacturer's instructions. Briefly, COS-7 cells transfected with GFP, clec14a-GFP, or clec14a $\Delta$ CTLG-GFP were added to each well of the wound healing insert. When cells were confluent, inserts were removed from the wells, and cells were washed twice with phosphate-buffered saline (PBS). Images were captured 20 h after wounding.

To analyze endothelial cell migration, HUVECs cultured in the insert until monolayer formation were incubated in the absence or presence of 20  $\mu$ g/ml clec14a-CTLG IgG or cetuximab for 9 h at 37 °C. Cells were washed twice with PBS and stained with crystal violet (Sigma, St Louis, MO, USA). For quantitative analysis, five fields per plate were photographed



**Figure 7.** Effect of clec14a-CTLD IgGs on clec14a-mediated cell-cell contact. (a) HEK293F cells transfected with GFP and clec14a-GFP were incubated in the absence (MOCK) or presence of clec14a-CTLD IgGs or cetuximab for 8 h. Cell aggregates (mass > 4 cells; arrowheads) were counted under a light microscope. The number of aggregates per field is shown in **b**. Values represent mean  $\pm$  s.d. of triplicate measurements from one of three independent experiments. (c) HEK293F cells transfected with GFP and clec14a-GFP were subjected to a cell viability assay with a CCK-8 cell counting kit. (d) Following preincubation of hCTLDFc with HUVECs, the extent of hCTLDFc binding to HUVECs in the absence or presence of increasing concentrations of clec14a-CTLD IgG (clone 1) was measured by cell ELISA. Values represent the mean  $\pm$  s.d. of triplicate measurements from one of three independent experiments.



**Figure 8.** Effect of clec14a-CTLDFc on CTLDFc-CTLDFc interaction. (a, b) COS-7 cells expressing GFP (white), clec14a-GFP (black) or clec14a $\Delta$ CTLDFc-GFP (light gray) were incubated with 0.15  $\mu$ g hCTLDFc or Fc and CTLDFc-CTLDFc interaction was measured by ELISA. Values represent mean  $\pm$  s.d. of triplicate measurements from one of three independent experiments.

under a light microscope (Nikon TS 100, Melville, NY, USA), and distance migrated was measured manually.

#### Immunocytochemistry

Immunocytochemistry was performed as described previously.<sup>22</sup> To measure filopodia formation, HUVECs were grown on collagen-coated coverslips for 1 day and transfected with GFP, clec14a-GFP or clec14a $\Delta$ CTLDFc-GFP. To measure cell morphology, HUVECs were incubated in the absence or presence of 20  $\mu$ g/ml clec14a-CTLDFc IgG (clone 1) for 1 day. Following fixation with 4% (w/v) paraformaldehyde for 30 min at 37  $^{\circ}$ C, cells were washed twice with PBS and blocked by incubation in PBS containing 5% (w/v) bovine serum albumin (BSA) and 0.1% Triton X-100 for 1 h at 37  $^{\circ}$ C. Cells were incubated with 0.1 unit/well rhodamine-phalloidin and/or 2  $\mu$ g/ml Hoechst for 1 h, and viewed under a confocal microscope (Olympus LX80 FV300, Olympus, Tokyo, Japan).

#### Construction and preparation of human and mouse CTLDFc fusion proteins

DNA encoding amino acid residues 31–172 of hCTLDFc was amplified using primers 5'-TCGCGCGCCGCTGCTCGGCCTCGGGGGCCTGC-3' and 5'-TCGCCTCGAGC TTGACACAGGTAGCCGTGG-3'. DNA encoding the same residues of mCTLDFc was amplified using primers 5'-TCGCGGCC CAGGCGGCTGTTCGGCCTCGGGG GCTTG-3' and 5'-TCGCGGCCGCGCTGG CCCTTGATAGGTAGCCATCGG-3'. PCR fragments were digested with *Sfi*I (NEB, Ipswich, MA, USA) and cloned into the modified mammalian expression vector pCEP4 (Invitrogen) encoding the hinge and CH2-CH3 domain of human IgG1 in the 3' region of the cloning site. Ligated products were transformed into competent *Escherichia coli* DH5 $\alpha$  cells and plasmid DNA was prepared.<sup>23</sup> HEK293F cells ( $6 \times 10^8$  cells) were transfected with 0.75 mg DNA each using 1.5 mg polyethylenimine (Polysciences, Inc., Warrington, PA, USA). After 7 days in culture, culture





according to manufacturer's instructions. Absorbance was measured at 450 nm with a VICTOR X4 spectrophotometer (Perkin Elmer).

### Measurement of cell-cell contact

Cell-cell contact assays were performed as described previously.<sup>13</sup> Briefly,  $1.5 \times 10^7$  HEK293F cells in suspension were transfected with plasmids expressing GFP, clec14a-GFP and clec14a $\Delta$ CTLG-GFP, cultured in Freestyle 293 expression medium overnight and seeded in six-well plates ( $5 \times 10^5$  cells/well). Cells were maintained in the absence or presence of 20  $\mu$ g/ml clec14a-CTLG IgG or cetuximab for 8 h. Cell aggregates (mass >4 cells) were counted in at least 10 fields and mean  $\pm$  s.d. were determined.

### Immunoblot analysis

Immunoblot analysis were performed as described previously.<sup>25</sup> Briefly, proteins were separated by electrophoresis in a 12% polyacrylamide gel and transferred to nitrocellulose membranes using a wet transfer system (GE Healthcare Life Sciences, Piscataway, NJ, USA). After blocking in tris-buffered saline and tween 20 (TTBS) buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.05 (v/v)% Tween 20) containing 5% (w/v) skimmed milk, the membranes were incubated with anti-GFP monoclonal antibody (1:5000; Santa Cruz Biotechnology, Dallas, TX, USA) or anti- $\beta$ -actin monoclonal antibody (1:5000; Applied Biological Materials, Richmond, BC, Canada) overnight at 4 °C. The membrane was washed with TTBS and incubated with HRP-conjugated Affinipure goat anti-mouse IgG (1:5000; Jackson ImmunoResearch) at room temperature for 1 h. Following several washes with TTBS, protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) according to manufacturer's instructions.

### Cell ELISA

For the competition assay with clec14a-CTLG IgG, HUVECs ( $10^4$ ) grown in 96-well plates were preincubated with 0.66  $\mu$ g of hCTLG-Fc and incubated in the absence or presence of increasing concentrations of clec14a-CTLG IgG (clone 1). Following three washes with PBS, bound hCTLG-Fc was measured using HRP-conjugated donkey anti-human Fc IgG (1:5000; Jackson ImmunoResearch).

To measure clec14a downregulation in HUVECs, HUVECs ( $10^4$ ) plated on 96-well plates were incubated with 20  $\mu$ g/ml clec14a-CTLG IgG or Fc for 0, 10, 30, 60, 120, or 180 min at 37 °C. Cells were washed twice with ice-cold PBS, blocked in 3% (w/v) BSA in PBS for 1 h at 4 °C, and incubated with sheep anti-clec14a polyclonal antibody (1:1000) in the blocking solution for 2 h at 4 °C. Then, cells were incubated with HRP-conjugated anti-sheep IgG (1:5000; Santa Cruz Biotechnology) for 1 h at 4 °C. After several washes with PBS, 100  $\mu$ l 3,3',5,5'-Tetramethylbenzidine substrate solution was added to each well. Optical density was measured at 450 nm using a microtiter plate reader.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### ACKNOWLEDGEMENTS

This work was supported by grants from Scripps Korea Antibody Institute and partly by Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2012R1A2A1A01002916).

### REFERENCES

- 1 Teillaud JL. From whole monoclonal antibodies to single domain antibodies: think small. *Methods Mol Biol* 2012; **911**: 3–13.
- 2 Zilian O. Scope for innovation in immunotherapy from the financial market's point of view: Phacilitate Immunotherapy Leaders' Forum 2012. *Hum Vaccin Immunother* 2012; **8**: 1370–1372.
- 3 Koch S, Claesson-Welsh L. Signal transduction by vascular endothelial growth factor receptors. *Cold Spring Harb Perspect Med* 2012; **2**: a006502.
- 4 Tortora G, Melisi D, Ciardiello F. Angiogenesis: a target for cancer therapy. *Curr Pharm Des* 2004; **10**: 11–26.
- 5 Bergsland E, Dickler MN. Maximizing the potential of bevacizumab in cancer treatment. *Oncologist* 2004; **9**(Suppl 1): 36–42.
- 6 Dienstmann R, Markman B, Tabernero J. Application of monoclonal antibodies as cancer therapy in solid tumors. *Curr Clin Pharmacol* 2012; **7**: 137–145.
- 7 Petit AM, Rak J, Hung MC, Rockwell P, Goldstein N, Fendly B *et al*. Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine

- kinases down-regulate vascular endothelial growth factor production by tumor cells *in vitro* and *in vivo*: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* 1997; **151**: 1523–1530.
- 8 Perrotte P, Matsumoto T, Inoue K, Kuniyasu H, Eve BY, Hicklin DJ *et al*. Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin Cancer Res* 1999; **5**: 257–265.
- 9 Vincenzi B, Schiavon G, Silletta M, Santini D, Tonini G. The biological properties of cetuximab. *Crit Rev Oncol Hematol* 2008; **68**: 93–106.
- 10 Huang SM, Li J, Harari PM. Molecular inhibition of angiogenesis and metastatic potential in human squamous cell carcinomas after epidermal growth factor receptor blockade. *Mol Cancer Ther* 2002; **1**: 507–514.
- 11 Kopetz S, Hoff PM, Morris JS, Wolff RA, Eng C, Glover KY *et al*. Phase II trial of infusional fluorouracil, irinotecan, and bevacizumab for metastatic colorectal cancer: efficacy and circulating angiogenic biomarkers associated with therapeutic resistance. *J Clin Oncol* **28**: 453–459.
- 12 Lucio-Eterovic AK, Piao Y, de Groot JF. Mediators of glioblastoma resistance and invasion during antivasculature endothelial growth factor therapy. *Clin Cancer Res* 2009; **15**: 4589–4599.
- 13 Rho SS, Choi HJ, Min JK, Lee HW, Park H, Kim YM *et al*. Clec14a is specifically expressed in endothelial cells and mediates cell to cell adhesion. *Biochem Biophys Res Commun* 2011; **404**: 103–108.
- 14 Mura M, Swain RK, Zhuang X, Vorschmitt H, Reynolds G, Durant S *et al*. Identification and angiogenic role of the novel tumor endothelial marker CLEC14A. *Oncogene* 2012; **31**: 293–305.
- 15 Carmeliet P. Angiogenesis in life, disease and medicine. *Nature* 2005; **438**: 932–936.
- 16 Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W *et al*. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *New Engl J Med* 2004; **350**: 2335–2342.
- 17 Gordon MS, Margolin K, Talpaz M, Sledge Jr. GW, Holmgren E, Benjamin R *et al*. Phase I safety and pharmacokinetic study of recombinant human anti-vascular endothelial growth factor in patients with advanced cancer. *J Clin Oncol* 2001; **19**: 843–850.
- 18 Kabbinnavar F, Hurwitz H, Fehrenbacher L, Meropol NJ, Novotny WF, Lieberman G *et al*. Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. *J Clin Oncol* 2003; **21**: 60–65.
- 19 Leow CC, Coffman K, Inigo I, Breen S, Czapiga M, Soukharev S *et al*. MEDI3617, a human anti-angiopoietin 2 monoclonal antibody, inhibits angiogenesis and tumor growth in human tumor xenograft models. *Int J Oncol* 2012; **40**: 1321–1330.
- 20 Lee SH. Tanibirumab (TTAC-0001): a fully human monoclonal antibody targets vascular endothelial growth factor receptor 2 (VEGFR-2). *Arch Pharm Res* 2011; **34**: 1223–1226.
- 21 Sprattlin J. Ramucirumab (IMC-1121B): Monoclonal antibody inhibition of vascular endothelial growth factor receptor-2. *Curr Oncol Rep* 2011; **13**: 97–102.
- 22 Lee S, Chung J, Ha IS, Yi K, Lee JE, Kang HG *et al*. Hydrogen peroxide increases human leukocyte adhesion to porcine aortic endothelial cells via NF- $\kappa$ B-dependent up-regulation of VCAM-1. *Int Immunol* 2007; **19**: 1349–1359.
- 23 Sambrook J, Russell DW. *Molecular cloning: a laboratory manual*. 3rd ed. (Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001).
- 24 Shukla AA, Hubbard B, Tressel T, Guhan S, Low D. Downstream processing of monoclonal antibodies—application of platform approaches. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007; **848**: 28–39.
- 25 Van Meter KE, Stuart MK. A monoclonal antibody that inhibits translation in Sf21 cell lysates is specific for glyceraldehyde-3-phosphate dehydrogenase. *Arch Insect Biochem Physiol* 2008; **69**: 107–117.
- 26 Barbas CF. *Phage display: a laboratory manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001.
- 27 Vestweber D. Lymphocyte trafficking through blood and lymphatic vessels: more than just selectins, chemokines and integrins. *Eur J Immunol* 2003; **33**: 1361–1364.
- 28 Alon R, Feigelson S. From rolling to arrest on blood vessels: leukocyte tap dancing on endothelial integrin ligands and chemokines at sub-second contacts. *Semin Immunol* 2002; **14**: 93–104.
- 29 Kim HY, Tsai S, Lo SC, Wear DJ, Izadjoo MJ. Production and characterization of chimeric monoclonal antibodies against Burkholderia pseudomallei and B. mallei using the DHFR expression system. *PLoS One* 2011; **6**: e19867.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/3.0/>

Supplementary Information accompanies this paper on the Oncogene website (<http://www.nature.com/onc>)