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Lung Tumor-Associated Dendritic Cell-Derived Amphiregulin Increased Cancer Progression

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The interaction of cancer within a microenvironment is an important factor determining cancer development. This study analyzed the soluble factors secreted by tumor-associated dendritic cells (TADCs), which are responsible for increasing lung cancer growth, migration, invasion, and epithelial-to-mesenchymal transition. Addition of amphiregulin, present in large amounts in TADC-conditioned medium (CM), mimicked the inductive effect of TADC-CM on lung cancer progression, supported by the enhancement of cell proliferation, migration, and invasion as well as osteolytic bone metastases phenotypes. In contrast, neutralization of amphiregulin from TADC-CM decreased the advanced malignancy-inductive properties of TADC-CM. Significant upregulation of amphiregulin has been seen in tumor-infiltrating CD11c+ DCs in human lung cancer samples and patients' sera. The enhancement of amphiregulin in TADCs has also been noted in mice transplanted with lung cancer cells. Induction of lung cancer progression by TADC-derived amphiregulin is associated with increased STAT3 and AKT activation, which subsequently increases the expression of cyclin D, Twist, and Snail. Blocking AKT significantly decreases TADC-CM and amphiregulin-mediated migration by decreasing the upregulation of Snail, whereas inhibition of STAT3 reduced the modulation of TADC-derived amphiregulin on Twist and cyclin D expression, suggesting that cooperation of STAT3 and AKT plays a critical role in TADC-mediated cancer progression. Moreover, mice treated with anti-amphiregulin Abs showed decreased incidence of cancer development and increased survival rates. Our study suggests that inhibition of amphiregulin or amphiregulin-related signaling is an attractive therapeutic target in lung cancer patients. The Journal of Immunology, 2011, 187: 000–000.
mobilization MAPK, or STAT3 signaling cascades (12, 14–16). AKT and STAT3 are known to be critical mediators in the control of cancer growth and progression. Therefore, we hypothesized that amphiregulin may play a role in mediating the regulatory effects of lung cancer development via the DCs–lung cancer cell interaction.

In this study, we discovered that TADCs secrete high amounts of amphiregulin and play a role in promoting cancer progression. Our data show that TADCs-derived amphiregulin increases A549 and H460 proliferation, migration, and epithelial-to-mesenchymal transition (EMT) by the paracrine effect. These findings also revealed that DCs infiltrating tumor tissues of mice and human patients express high levels of amphiregulin, suggesting a novel role of amphiregulin in the interaction of cancer and DCs. Furthermore, treatment of mice with anti-amphiregulin Abs decreased the incidence of lung tumors and increased survival rates. Investigating the mechanisms by which TADCs contributes to lung cancer progression may facilitate the discovery of a potential target for developing therapeutic strategies targeting immune cells in the tumor microenvironment.

Materials and Methods

Lung cancer cells and conditioned media

Human lung cancer cells A549 and H460 and mouse Lewis lung carcinoma (LLC) cells were obtained from the Bioresource Collection and Research Center (Hsinchu City, Taiwan). To obtain the A549 and H460-conditioned medium (CM), cells were seeded at 2 × 10^5 cells/100 mm dish and cultured for 24 h. The medium was replaced, and the supernatants were harvested after 48 h of incubation.

Serum samples from lung cancer patients

Preoperative blood samples were obtained from 62 lung cancer patients and 19 healthy donors admitted to the Division of Pulmonary and Critical Care Medicine, Kaohsiung Medical University Hospital (Kaohsiung, Taiwan). Serum was separated by centrifugation and frozen at −80°C. Approval for these studies was obtained from the Institutional Review Board of Kaohsiung Medical University Hospital. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Measurement of secreted factors

Supernatants from TADC, monocyte-derived DCs (mdDCs), and A549 and H460 cells were collected. IL-6 was assessed by IL-6 ELISA kit. Amphiregulin and IL-8 levels were quantified using the DuoSet ELISA. MMP-2 and MMP-9 levels were determined by gelatin zymography.

Isolation of CD14+ monocytes and differentiation of mdDCs

Monocytes were purified from PBMCs obtained from healthy consenting donors. Mononuclear cells were isolated by ficoll-hypaque gradient (GE Healthcare Bio-Sciences, Little Chalfont, U.K.). CD14+ monocytes were purified using CD14+ mAb-conjugated magnetic beads (MACS MicroBeads; Miltenyi Biotec), according to the manufacturer’s protocol. mdDCs were generated by cultivating CD14+ monocytes in RPMI 1640 medium containing 10% FBS (Invitrogen, Carlsbad, CA) and 20 ng/ml GM-CSF and 10 ng/ml IL-4 (R&D Systems, Minneapolis, MN) for 5 d. The medium was replaced every 3 d. Osteoclast differentiation and activity assay

PBMCs were plated and incubated overnight at 37°C. Nonadherent cells were removed by washing with PBS, and the remaining adherent cells were grown in culture medium containing mdDC-CM, A549-TADC-CM, H460-TADC-CM, or amphiregulin (10 ng/ml) in 200 ng/ml human M-CSF and 100 ng/ml human RANKL for 7 d. The medium was replaced every 3 d. Osteoclast formation was measured by quantifying cells positively stained by TRAP (Sigma-Aldrich, St. Louis, MO); osteoclasts were determined to be TRAP-positive staining multinuclear (more than three nuclei) cells by means of light microscopy. The TRAP-positive cells, and the number of nuclei per TRAP-positive cell in each well, were counted. Osteoclasts’ bone resorption activity was assessed by BD BioCoat Osteogenic Bone Resorption assay (BD Biosciences, Bedford, MA), in the same culture conditions as described above.

Gene knockdown by small interfering RNA

Monocytes were transfected with 1 μmol/l nontarget or amphiregulin small interfering RNA (siRNA) pool (Dharmacon) in Accell delivery media (B-005000), according to the manufacturer’s instructions. Positive controls Accell GAPDH siRNA and scrambled Accell siRNA pool were used in the experiments. After 72 h transfection, the medium was changed to RPMI 1640 medium containing 10% FBS, 20 ng/ml GM-CSF and 10 ng/ml IL-4 presenting in A549-CM and H460-CM, and then stimulated by IFN-γ for 48 h. Nontarget or amphiregulin Accell siRNAs were added every 3 d to maintain the knockdown efficiency of siRNA. The supernatants of mdDCs were collected as CMs. The amphiregulin changes of mdDCs were measured by real-time PCR as described above.

Analysis of cell proliferation, migration, and invasion

Cell proliferation was assessed by Premixed WST-1 Cell Proliferation Analysis kit (BD Biosystems, Foster City, CA). Each PCR mixture contained 200 ng of each primer, 10 μl 2× SYBR Green PCR Master Mix (Applied Biosystems), and 50 ng of DNA and RNase-free water, with a total volume of 20 μl. The PCR was carried out with a denaturation step at 95°C for 10 min and then for 40 cycles at 95°C for 15 s and at 60°C for 1 min. All PCRs were performed in triplicate and normalized to internal control GAPDH mRNA. Relative expression was presented using the 2−ΔΔCT method.

Immunofluorescence

Noncancerous lung tissue specimens obtained from human lung cancer patients were embedded in OCT and frozen in liquid nitrogen. Sections (3–5 μm) were fixed with acetone at −20°C and then stained with amphiregulin Ab (Abcam) or anti-CD11c Ab. After a wash with PBS containing 0.1% Tween 20, slides were incubated with Dylight 488- or Dylight 549-conjugated secondary Ab (Rockland, Gilbertsville, PA), with or without DAPI, for 1 h at room temperature. The data were analyzed with a confocal laser scanning microscope (Fluoview FV500; Olympus, Tokyo, Japan).

Quantitative real-time PCR

RNA isolation was performed using the TRIzol reagent (Invitrogen). cDNA was prepared using an oligo(dT) primer and reverse transcriptase (Takara, Shiga, Japan) following standard protocols. Real-time PCR was performed using SYBR Green on the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Each PCR mixture contained 200 ng of each primer, 10 μl 2× SYBR Green PCR Master Mix (Applied Biosystems), and 50 ng of DNA and RNase-free water, with a total volume of 20 μl. Relative expression was presented using the 2−ΔΔCT method.

Immunoblot

Cells were lysed on ice for 15 min by M-PEL lysis reagent (Pierce). Cell lysate was centrifuged at 14,000 × g for 15 min, and the supernatant fraction was collected for immunoblot. Equivalent amounts of protein were resolved by SDS-PAGE (8–12%) and transferred to polyvinylidene difluoride membranes. After blocking for 1 h in 5% nonfat dry milk in TBS, the membrane was incubated with the desired primary Ab for 1–1.6 h. The membrane was then treated with appropriate peroxidase-conjugated secondary Ab, and the immunoreactive proteins were detected using an ECL kit (Millipore), according to the manufacturer’s instructions.
the limbs and then placed in RPMI 1640 containing murine GM-CSF (20 ng/ml) and murine IL-4 (20 ng/ml, R&D Systems), with or without LLC-CM, for 24 h. The expression of various mRNAs was assessed by real-time PCR. Survival rates were evaluated in mice injected with LLC cells. Iso-
type control Ig (IgG) and amphiregulin (50 μg/mice) Ab were i.p. injected every 7 d three times after administration of LLC. Animals were sacrificed on day 24 after LLC cell transplantation and the number of tumor nodules recorded for the analysis of lung cancer incidence. For survival assay, all animals were closely monitored daily for signs of distress and were sac-
rificed when they appeared lethargic or to have increased breathing rates.

**Statistical analysis**

Data were expressed as means ± SD. Statistical comparisons of the results were made using ANOVA. Significant differences (p < 0.05) between the means of the two test groups were analyzed by Student t test. Survival studies were assessed using Kaplan–Meier survival curves and analyzed with the Mantel–Cox log-rank test.

**Results**

**TADCs increased lung cancer cell proliferation, migration and EMT**

To investigate the influence of TADCs on lung cancer progression, we assessed the effect of TADC-CM on lung cancer cell proliferation, migration, and invasion. As shown in Fig. 1A, A549-
TADC-CM (20%) and H460-TADC-CM (20%) increased the proliferation of A549 and H460 cells after 72 h of treatment. In addition, both A549-TADC-CM and H460-TADC-CM increased the migration and invasive abilities of A549 and H460 cells (Fig. 1B, 1C). Furthermore, A549-TADC-CM and H460-TADC-CM also caused A549 and H460 cells to undergo EMT, including the downregulation of epithelial markers (E-cadherin, claudin-3, and ZO-1), and upregulation of fibroblast makers (N-cadherin, vimentin, and fibronectin) (Fig. 1D).

The effects of TADC on lung cancer migration and invasion were also confirmed by coculture system. As shown in Supplemental Fig. 1B and 1C, coculture of A549 or H460 cells with A549-
TADCs or H460-TADCs enhances the migratory and invasive ca-
pability of A549 and H460 cells, respectively. These coculture system data coincide with that of TADC-CM treatment.

**Amphiregulin plays an important role in TADCs-mediated cancer progression**

To investigate which factor is responsible for TADCs-mediated lung cancer development, we used a microarray to assess the gene profile of A549-TADCs. The data showed that several soluble factors were upregulated in A549-TADCs when compared with mDCs. Among these upregulated genes, levels of amphiregulin, a lung cancer-related growth factor, increased 11.73-fold in A549-
TADCs (Fig. 2A). In addition, ADAM17, an enzyme responsible for amphiregulin release, was also increased in A549-TADCs. Quantitative real-time PCR (Q-PCR) analysis further revealed that A549-CM or H460-CM increased the expression of amphiregulin in A549-TADCs and H460-TADCs (Fig. 2A). Furthermore, protein levels of amphiregulin were enhanced in A549-TADCs and H460-TADCs, as determined by ELISA analysis (Supple-
mental Fig. 1D).

We also assessed the effect of amphiregulin protein on the proliferation, migration, invasion, and EMT of A549 and H460 cells. Amphiregulin increased the proliferation of A549 and H460 cells in a concentration-dependent manner (Supplemental Fig. 1E). Moreover, amphiregulin not only increased the migratory ability of A549 and H460 (Fig. 2B) but also enhanced their invasive ability and EMT (Fig. 2C, 2D).

To better understand the role of amphiregulin, we blocked the effect of amphiregulin on A549 and H460 proliferation and mi-

**TADC-derived amphiregulin stimulated lung cancer to produce bone metastasis factor (IL-6, IL-8 and parathyroid hormone-related protein) and osteoclastogenesis**

Bone is a frequent target of lung cancer metastasis, and bone metastasis is mediated by complex interactions between tumor cells and resident stromal cells in the cancer microenvironment (18). We therefore assessed the potential role of TADC-derived amphiregulin in lung cancer metastasis. As shown in Fig. 5A and 5B, A549-TADC-CM and H460-TADC-CM increased A549 or H460 cells’ expression of two inflammatory cytokines, IL-6 and IL-8, which have been implicated in bone metastasis (19, 20). Similar to TADC-CM, amphiregulin also increases the expression of IL-6 and IL-8 in both A549 and H460 cells. PTHrP, a causative factor that contributes to osteolytic metastases, is also regulated in both A549-TADC-CM or amphiregulin-treated A549 and H460-TADC-CM or amphiregulin-treated H460 cells (Fig. 5C).

Amphiregulin also increases the formation and activity of osteo-
clasts from PBMCs presenting in M-CSF and RANKL (Fig. 5D). In addition, knockdown of amphiregulin in monocytes by siRNA also decreases the upregulation of TADC-CMs on PTHrP expression in both A549 and H460 cells (Fig. 5E).

**STAT3 and AKT activation are involved in the amphiregulin-mediated effect on lung cancer**

Because TADC-derived amphiregulin increased cell proliferation, migration, and invasion, we assessed the effect of TADC-CMs and amphiregulin on several oncogenic signaling pathways, including AKT and STAT3 (15, 16). The results showed that exposure of A549 and H460 cells to A549-TADC-CM or H460-TADC-CM led to the phosphorylation of STAT3 and AKT. In contrast, the expression of STAT3 and AKT (unphosphorylated form) was un-
altered by TADC-CM treatment in either A549 or H460 cells (Fig. 6A). Similar results were also found in amphiregulin-treated A549 and H460 cells (Fig. 7A). In addition, both TADC-CMs and amphiregulin increased the expression of Sna1 and Twist, two EMT-related transcriptional factors, and enhanced the levels of cyclin D in both A549 and H460 cells (Figs. 6A, 7A).

To investigate the functional impact of AKT and STAT3 acti-
vation on TADC-derived amphiregulin in lung cancer, we used chemical inhibitors Ly294002 and AG490 to specifically inhibit AKT and STAT3 signaling, respectively. STAT3 inhibitor AG490 decreased TADC-CM and amphiregulin-mediated cell prolifera-
tion, migration, and invasion (Figs. 6B–D, 7B–D). In contrast, AKT inhibitor Ly294002 markedly inhibited two types of TADC-CMs and amphiregulin-induced cell migration in both A549 and H460 cell lines, whereas it only slightly decreased TADC-CM and amphiregulin-driven cell proliferation. Furthermore, pretreatment of A549 and H460 cells with STAT3 inhibitor completely
abrogated expression of Twist and cyclin D but only partially decreased Snail caused by A549-TADC-CM, H460-TADC-CM, and amphiregulin (Figs. 6E, 7E). In contrast, AKT inhibitor completely inhibited Snail expression, but not Twist and cyclin D. Moreover, both STAT3 and AKT inhibitors reversed the inhibitory effect of TADC-CMs on E-cadherin in A549 and H460 cells. These data suggest that STAT3 and AKT play important roles in lung cancer progression (Figs. 6E, 7E).

High amounts of amphiregulin in CD11c+ DCs are found in the tumor sections of lung cancer-bearing mice and human lung cancer patients

To confirm the role of amphiregulin on TADC-mediated cancer progression in humans, we characterized the expression of amphiregulin in tumor-infiltrated CD11c+ DCs. Immunofluorescence staining of the marginal regions of a patient’s lung cancer reveals the presence of many CD11c+ DCs, which have infiltrated...
the area around the tumor. These areas express higher levels of amphiregulin in comparison with tissue sections of non-tumor regions (Fig. 8A). Furthermore, a marked statistical difference has also been found between lung cancer patients and healthy donor serum (Fig. 8B).

We also used animal experiments to determine whether lung cancer increased amphiregulin expression in DCs in vivo. We injected mouse lung cancer cell line LLC into mice and then allowed the cells to develop for 14 d. LLC-CM increased the production of amphiregulin in mice bone marrow cells presenting in IL-4 and GM-SCF (Supplemental Fig. 3A). These data coincide with human lung cancer data. Q-PCR and ELISA analysis have also shown that lung tumor-infiltrated CD11c+ DCs produce elevated levels of amphiregulin in mRNA and protein levels (Fig. 8C, Supplemental Fig. 3B).

Amphiregulin Ab decreased cancer metastasis in vivo
Finally, we assessed whether inhibiting amphiregulin production from tumor-infiltrated DCs could decrease lung cancer development. Treatment of mice by anti-amphiregulin mAb inhibited the growth of LLC in 25% (two of eight) of mice, in comparison with 85% (six of seven) of rat IgG-treated control mice (Fig. 8D). Furthermore, treatment by anti-amphiregulin mAb also improved long-term survival of mice (66.6%) with LLC tumors (up to 90 d), whereas all mice treated by control IgG died of cancer within 25 d (Fig. 8E).

Discussion
The microenvironment of a tumor is widely known to be an important factor regulating cancer development. Cells surrounding
AMPHIREGULIN INVOLVED IN CANCER–IMMUNE SYSTEM INTERACTION

It is of interest to note that amphiregulin-producing CD11c+ DCs were found to infiltrate the cancereous lung tissue of LLC-bearing mice. In addition, CD11c+ DCs located in human lung cancer tissues also produce high amounts of amphiregulin when compared with CD11c− DCs in nontumorous regions. Amphiregulin levels in lung cancer patients’ serum are also higher than those of healthy donors. Furthermore, antagonism of amphiregulin by injection of specific Abs decreases the recurrence of LLC and increases the survival rates of LLC-bearing mice. These results from experimental cell studies, mouse models, and clinical patient sections strongly suggest that amphiregulin is an important effector molecule through which TADCs enhance the development of lung cancer.

STAT3 signaling has been demonstrated to participate in various cellular processes, including the inflammatory response, cell proliferation, differentiation, survival, and motility (28). STAT3 is activated by multiple receptor and nonreceptor tyrosine kinases in response to various cytokines, hormones, and growth factors, and abnormal activation of STAT3 signaling has been seen in multiple tumors (28, 29). STAT3 has been reported to be involved in epidermal growth factor-stimulated cancer proliferation and migration by increasing cyclin D and Twist expression, respectively (16, 30). Twist is the basic helix-loop-helix transcription factor essential for regulating EMT of cancer by decreasing E-cadherin expression and is upregulated in a number of human tumors, including lung cancer (31). In this study, we have shown that treatment of A549 and H460 with TADC-CM and amphiregulin resulted in increased STAT3 phosphorylation and Twist expression. Inhibition of STAT3 activity by specific inhibitor decreases the effect of TADC-CM on cell proliferation (A), migration (B), and invasion (C). A549 and H460 cells were treated with mDC-CMs or TADC-CMs presenting in amphiregulin Ab or IgG. Cell proliferation was assessed by WST-1 assay (72 h incubation). Lung cancer cells were seeded in the upper insert and were treated with or without amphiregulin Ab (2 μg/ml) or IgG (2 μg/ml), and TADC-CMs (20%) was added into the lower well to act as an attractive agent for 24 h. The migratory and invasive cells were quantified as described above. Each value is the mean ± SD of three independent experiments. The asterisk indicates a significant difference between the two test groups, as analyzed by Student t test (p < 0.05).

FIGURE 3. Neutralizing amphiregulin by its specific Ab decreased TADC-CM–mediated cancer progression. Amphiregulin Ab decreased the effect of TADC-CM on cell proliferation (A), migration (B), and invasion (C). A549 and H460 cells were treated with mDC-CMs or TADC-CMs presenting in amphiregulin Ab or IgG. Cell proliferation was assessed by WST-1 assay (72 h incubation). Lung cancer cells were seeded in the upper insert and were treated with or without amphiregulin Ab (2 μg/ml) or IgG (2 μg/ml), and TADC-CMs (20%) was added into the lower well to act as an attractive agent for 24 h. The migratory and invasive cells were quantified as described above. Each value is the mean ± SD of three independent experiments. The asterisk indicates a significant difference between the two test groups, as analyzed by Student t test (p < 0.05).
cyclin D and Twist expression, suggesting that STAT3 is an upstream regulator of cyclin D and Twist. Moreover, STAT3 inhibitor decreases the effects of TADC-CM and amphiregulin on bone metastasis factor PThrP production and cell migration in A549 and H460 cells, suggesting that the activation of STAT3 signaling plays a crucial role in the development of lung cancer growth and metastasis.

AKT, a downstream effector of PI3K has been shown to be a critical mediator of cell proliferation, survival, and metastasis in a variety of cell types. AKT has been reported to act as an important factor in amphiregulin-mediated cell survival and migration (32, 33). In our study, we found that treating A549 and H460 cells with TADC-CM or amphiregulin dramatically increases the activation of AKT. The inductive effect of TADC-
FIGURE 5. TADC-derived amphiregulin increased bone metastasis-related factors in lung cancer. A. TADCd and amphiregulin increased the production of IL-6 (A), IL-8 (B), and PTHrP (C) in A549 and H460 cells. A549 and H460 cells were treated with A549-TADC-CM (20%) or H460-TADC-CM (20%) and amphiregulin (10 ng/ml) for 24 h, and then, the supernatants were collected and assessed by ELISA kits. D. Amphiregulin increased osteoclast differentiation and activity. PBMCs were treated with amphiregulin (10 ng/ml) presenting in RANKL (100 ng/ml) and M-CSF (200 ng/ml) for 7 d. Osteoclast cells were stained for TRAP activity, and bone resorption activity was determined by the Osteologic MultiTest Slide. E. Inhibition of amphiregulin decreased the enhancement of TADCs on PTHrP expression. A549 and H460 cells were treated with various amphiregulin siRNA or scrambled siRNA-transfected A549 or H460-associated DC-CMs (20%) for 24 h, and then, the cells’ supernatants were collected and assessed by PTHrP ELISA kits. Each value is the mean ± SD of three independent experiments. The asterisk indicates a significant difference with control, as analyzed by Student t test (*p < 0.05).
FIGURE 6. TADC-CM promote cancer progression by STAT3 and AKT activation. A, TADC-CM increased STAT3 and AKT activation and enhanced Snail and Twist expression. Cells were treated with TADC-CM (20%) for the indicated times, and the expression of various proteins was assessed by immunoblot assay. STAT3 and AKT inhibitors decreased TADC-mediated cell proliferation (B), migration (C), invasion (D), and EMT (E). Cells were pretreated with or without AKT (5 μM) or STAT3 (1 μM) inhibitor and then cultured with TADC-CM (20%) for the indicated times (72 h for cell proliferation, 24 h for E-cadherin, and 6 h for Snail, Twist, and cyclin D). Cell proliferation was assessed by WST-1 assay. The invasiveness and migration ability of A549 and H460 cells were quantified by QCM 24-well Cell Migration and Invasion assay. Cells were seeded in the upper inserts were treated with or without AKT and STAT3 inhibitors, with amphiregulin acting as a chemoattractant for cancer migration and invasion. Each value is the mean ± SD of three independent experiments. The asterisk indicates a significant difference between the two test groups, as analyzed by Student t test (*p < 0.05).
CM or amphiregulin on AKT is responsible for the increase of Snail expression, a repressor of epithelial type marker E-cadherin. Furthermore, selective inhibition of AKT by chemical inhibitor also decreases the effects of TADC-CM or amphiregulin on cell migration and invasion, suggesting that phenotypic transition of lung cancer by TADC involves amphiregulin-mediated

**FIGURE 7.** Amphiregulin promotes cancer progression by STAT3 and AKT activation. A, Amphiregulin increased STAT3 and AKT activation and enhanced Snail and Twist expression. Cells were treated with amphiregulin (10 ng/ml) for the indicated times, and the expression of various proteins was assessed by immunoblot assay. STAT3 and AKT inhibitors decreased amphiregulin-mediated cell proliferation (B), migration (C), invasion (D), and EMT (E). Cells were pretreated with or without AKT or STAT3 inhibitor and then cultured with amphiregulin (10 ng/ml) for the indicated times (72 h for cell proliferation, 24 h for E-cadherin, and 6 h for Snail, Twist, and cyclin D). Cell proliferation, invasiveness, and migration ability of A549 and H460 cells were quantified by as described above. Each value is the mean ± SD of three independent experiments. The asterisk indicates a significant difference between the two test groups, as analyzed by Student t test (*p < 0.05).
FIGURE 8. Elevated amounts of amphiregulin on CD11c+ DCs in the lung cancer-bearing patient and mice. A, Amphiregulin\textsuperscript{high}D11c+ DCs infiltrated cancer sections. Nontumor and tumor regions (n = 15) were cut and stained and then samples were analyzed by confocal microscopy (original magnification ×40). Arrows indicate the CD11c+/amphiregulatin+ cells. B, High levels of amphiregulin were found in the serum of lung cancer patients. Amphiregulin levels of lung cancer patients’ (n = 62) and healthy donors’ sera (n = 19) were assessed by ELISA. C, Elevated levels of amphiregulin were found in CD11c+ DCs isolated from the lungs of lung-cancer bearing mice. Culture media and LLC were injected into mice via the tail vein (control group, n = 10; LLC group, n = 8). After 24 d, nontumorous and tumorous regions of the lungs were harvested. CD11c+ DCs were isolated from fresh lung or tumor tissue, and amphiregulin levels were assessed by Q-PCR. Neutralization of amphiregulin decreased the occurrence of lung cancer (D) and improved survival rates (E) in mice. LLCs were injected into mice via the tail vein. Mice were dosed every 7 d with i.p. injections IgG (n = 7) or amphiregulin Abs (50 µg/ml) (n = 8). Arrows indicate the time of administration of amphiregulin Ab. The asterisk indicates a significant difference with the control, as analyzed by ANOVA with Student t test post hoc (*p < 0.05).
activation of AKT and subsequent AKT-activated Snail gene expression.

Taken together, our findings suggest that a new mechanism of TADCs promotes lung cancer progression, in which TADC-derived amphiregulin causes phenotypic transition and reinforces cancer progression. Amphiregulin enhances the cooperation of STAT3 and AKT signaling and subsequently increases the expression of Twist and Snail, resulting in the promotion of lung cancer development. Neutralization of amphiregulin by amphiregulin Ab significantly decreases the incidence of cancer development in mice. Our findings provide important insights into the biology of tumors with vicious interactions of DCs and cancer as well as establish new rationales for using anti-amphiregulin strategies to target aggressive invasive lung cancer.

Disclosures

The authors have no financial conflicts of interest.

References