

The effects of BAFF and APRIL signaling on non-small cell lung cancer cell proliferation and invasiveness

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Abstract. Lung cancer represents the most common type of human malignancy and is the main cause of cancer-associated mortality worldwide. To improve the effectiveness of treatment strategies, a better understanding of the mechanisms of cancer progression and invasiveness is required. Recently, B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), two relatively newly described cytokines belonging to the tumor necrosis factor superfamily, have been shown to play a role in cancer progression. However, at present, the effects of both cytokines on lung cancer cells remain unclear. The present study aimed therefore to understand the direct effects of BAFF and APRIL on non-small cell lung cancer (NSCLC) progression. To do so, reverse transcription quantitative PCR and western blotting were used to evaluate whether A549 and H2030 NSCLC cells express receptors for both BAFF and APRIL. The results demonstrated that both investigated cell lines expressed BAFF-R (receptor specific to BAFF only) and transmembrane activator and CAML interactor (TACI; shared receptor for both cytokines). In addition, functional experiments were performed to determine the effects of BAFF and APRIL stimulation on cancer cell

viability. The results demonstrated no direct effects of BAFF and APRIL on NSCLC cell proliferation and invasiveness. In summary, the present study demonstrated that NSCLC cells possess the ability to respond directly to both BAFF and APRIL. However, activation of BAFF-R and TACI signaling in cancer cells did not increase the proliferative capacity and invasiveness. Further investigation is thus required to better understand the role of BAFF and APRIL on the progression of NSCLC.

Introduction

Lung cancer is the most common malignancy diagnosed worldwide and represents the leading cause of cancer associated-mortality. Among all types of lung cancer, non-small cell lung cancer (NSCLC) is the most frequent, accounting for ~85% of all lung cancer cases (1). Although lung neoplasm pathogenesis is known to rise constitutively, it is crucial to better understand the mechanisms of cancer progression, including mutual interactions between malignant and immune cells (2,3). Due to immune regulatory properties, cancer cells regulate innate and adaptive immune responses, abolishing antitumor activities and supporting mechanisms promoting tumor progression (4,5). Among tumor-infiltrating immune cells, the vast majority is represented by myeloid cells, such as monocytes/macrophages, which are referred to as tumor-associated macrophages (6,7). Due to pleiotropic biological activities, myeloid cells play a central role in the regulation of the tumor microenvironment by means of secreted soluble factors, including proteins belonging to the tumor necrosis factor (TNF) superfamily (8,9).

B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) are relatively newly discovered TNF superfamily members. Both ligands might act through direct interaction with shared receptors, such as B-cell maturation antigen (BCMA) and transmembrane activator and CAML interactor (TACI), while BAFF is also explicitly recognized by BAFF receptor (BAFF-R). Both ligands serve a crucial role in B cell development, maturation and immunoglobulin class switching (10). Furthermore, BAFF was shown to stimulate anti-apoptotic signaling in tumor cells (8,11). Previous

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studies demonstrated that BAFF could play a role in neoplasm progression and aggressiveness (12-14). In addition, it was reported that both BAFF and APRIL signaling might increase tumor cell proliferation and enhance tumor cell viability in response to chemotherapeutic drugs in hematopoietic malignancies (8,9,15).

At present, to the best of our knowledge, the role of BAFF and APRIL in solid tumors development and progression remains unknown. However, the expression of their receptors was mainly reported in tumor-infiltrating cells (16,17). Interestingly, elevated blood levels of BAFF and APRIL are associated with a higher stage of the disease and cancer invasiveness, such as in breast cancer, chronic lymphocytic leukemia and pancreatic cancer (12,18). Furthermore, the elevated expression of BAFF and APRIL is associated with increased cell migration, epithelial-mesenchymal transition and stemness, subsequently contributing to an aggressive phenotype of breast cancer (12,19,20). Similarly, APRIL overexpression was shown to be associated with tumor progression and was therefore proposed as a potential prognostic factor in rectal cancer, pancreatic adenocarcinoma and various B-cell malignancies (13). In addition, in hepatocellular carcinoma cells, APRIL can play pleiotropic biological activities. Subsequently, APRIL may support tumor growth or reduce cell proliferation, depending on the particular pathway activation (21). Previous studies reported for the first time the importance of BAFF and APRIL signaling in NSCLC (22,23). However, to date, it remains unclear whether BAFF and APRIL may serve a direct role in regulating lung cancer cell proliferation and invasiveness (22,24). The present study aimed therefore to evaluate the effects of BAFF and APRIL on NSCLC cell proliferation and invasiveness.

Materials and methods

Cell lines. The human NSCLC cell lines A549 and H2030 were purchased from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (PAN Biotech UK, Ltd.) supplemented with 10% of heat-inactivated and filtered FBS (PAN Biotech UK, Ltd.) and gentamycin (50 µg/ml; Gibco; Thermo Fisher Scientific, Inc.) and placed at 37°C in a humidified incubator containing 5% CO₂. Cells were passaged when at 70-80% confluence. Cells from the second to fifth passage were used for all experiments.

Reverse transcription quantitative PCR. To assess the expression level of BAFF, APRIL, BAFF-R, BCMA and TACI, cells were harvested, washed with PBS and lysed in RLT buffer (Qiagen) supplemented with 1% β-mercaptoethanol (Sigma-Aldrich; Merck KGaA) at room temperature. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturers' protocol and quantified using NanoDrop (NanoDrop 2000c/2000; Thermo Fisher Scientific, Inc.). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.) according to manufacturers' instructions. Commercially available TaqMan assays (Thermo Fisher Scientific, Inc.; Table I) and TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Inc.) were used according to manufacturers' instructions. The thermocycling conditions

were as follows: Polymerase activation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. Samples were analyzed in the StepOne Plus system (Thermo Fisher Scientific, Inc.). Data were analyzed using StepOne Software v2.3 (Thermo Fisher Scientific, Inc.). The relative expression levels were normalized to endogenous control and were expressed as 2^{-ΔΔC_q} (25).

Western blotting. To evaluate the protein expression of BAFF, APRIL, BAFF-R, BCMA and TACI, cells were harvested, washed in PBS and lysed in RIPA buffer (Thermo Fisher Scientific, Inc.) supplemented with complete protease inhibitor cocktail (Roche Diagnostics) for 15 min on ice. The cell debris were removed by centrifugation at 13,000 x g for 5 min at 4°C. The concentration of total protein was assessed using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.) according to manufacturers' instructions. Proteins (20 µg) were mixed with Lamli sample buffer (1:4; Bio-Rad Laboratories, Inc.) and heated at 95°C for 5 min. Proteins were separated on 4-10% TGX gel (Bio-Rad Laboratories, Inc.) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.) using the TransBlot turbo system (Bio-Rad Laboratories, Inc.). The membranes were blocked using 5% skimmed milk dissolved in PBS and supplemented with 0.1% Tween-20 (Sigma-Aldrich; Merck KGaA; T-PBS) for 1 h at room temperature. Membranes were incubated with primary antibodies overnight at 4°C (Table II). Subsequently, membranes were washed using 10X PBS (Corning) supplemented with 0.1% Tween-20 and incubated with specific HRP-conjugated secondary antibodies (Table II) for 1 h in room temperature. Bands were detected using the SuperSignal West Femto chemiluminescence substrate kit (Thermo Fisher Scientific, Inc.) and visualized with ChemiDoc Touch System (Bio-Rad Laboratories, Inc.). Relative expression levels were normalized to endogenous control (β-actin) using ImageJ software v1.53 (National Institutes of Health).

Ligand receptor interaction blocking. To block the interactions of BAFF, APRIL and their receptors in A549 and H2030 cell lines, blocking antibodies for TACI (monoclonal mouse IgG1 clone #165609; R&D Systems, Inc.; cat. no. MAB174) and BAFF-R (monoclonal mouse IgG2a, kappa clone #8A7; Thermo Fisher Scientific, Inc.) were used separately or in combination at 10 µg/ml for 24 h in standard cell culture conditions (37°C, 5% CO₂).

Cell proliferation assay. To assess the effects of BAFF and APRIL on lung cancer cell proliferation, the cells were harvested and labeled with carboxyfluorescein succinimidyl ester (CFSE; Sigma-Aldrich; Merck KGaA). The cells were cultured for 72 h in 24 well plates (37°C, 5% CO₂) in the presence of recombinant human BAFF or APRIL proteins at the concentrations of 50, 100 or 150 ng/ml or without any stimulation (vehicle). The cells were acquired by using TrypLE Select (Thermo Fisher Scientific, Inc.) at 24, 48 and 72 h. Subsequently, cells were washed in PBS and stained with 7-AAD as suggested by the manufacturer (0.25 µg per 1x10⁶ cells; Becton-Dickinson and Company), and incubated

Table I. TaqMan assays used for reverse transcription quantitative PCR.

Assay	Target species	Catalogue number	Manufacturer
TNFSF13 (BAFF)	Human	Hs00198106_m1	Thermo Fisher Scientific, Inc.
TNFSF13 (APRIL)	Human	Hs00601664_g1	Thermo Fisher Scientific, Inc.
TNFRSF1 (BAFF-R)	Human	Hs00606874_g1	Thermo Fisher Scientific, Inc.
TNFRSF1 (TACI)	Human	Hs00963364_m1	Thermo Fisher Scientific, Inc.
TNFRSF1 (BCMA)	Human	Hs03045080_m1	Thermo Fisher Scientific, Inc.
GADPH	Human	Hs02786624_g1	Thermo Fisher Scientific, Inc.

APRIL, a proliferation-inducing ligand; BAFF, B-cell activating factor; BAFF-R, BAFF receptor; TACI, transmembrane activator and CAML interactor; BCMA, B-cell maturation antigen.

Table II. Description of antibodies used for western blotting.

Antibody	Target species	Host	Isotype	Clone	Dilution	Catalogue number	Manufacturer
BAFF-R	Human, mouse and rat	Mouse	IgG2b	H-1	1:150	sc-365410	Santa Cruz Biotechnology, Inc.
TACI	Human, mouse and rat	Mouse	IgG2b	C-9	1:200	sc-365253	Santa Cruz Biotechnology, Inc.
BCMA	Human	Rat	IgG2a	335004	1:100	MAB193	R&D Systems, Inc.
β-actin (Direct-Blot HRP)	Human, mouse and rat	Mouse	IgG2b	2F1-1	1:1000	643807	BioLegend, Inc.
Anti-mouse IgG HRP-conjugated	Mouse	Goat	IgG	Polyclonal	1:1000	HAF007	R&D Systems, Inc.
Anti-rat IgG HRP-conjugated	Rat	Goat	IgG	Polyclonal	1:1000	HAF005	R&D Systems, Inc.

BAFF-R, B-cell activating factor receptor; TACI, transmembrane activator and CAML interactor; BCMA, B-cell maturation antigen. HRP, horseradish peroxidase; Ig, immunoglobulin.

for 15 min at 4°C. Cells were analyzed on FACSCalibur flow cytometry system (Becton-Dickinson and Company). Data were analyzed using FlowJo software version 10.6.1 (Tree Star, Inc.). To set the gates for the analysis of proliferation (negative control), colcemid (50 ng/ml) was used to arrest the mitosis in metastasis stage. Cell viability was assessed according to 7-AAD staining and viable cells were considered as 7-AAD negative.

Invasion assay. To evaluate the influence of BAFF and APRIL on non-small cell lung cancer invasiveness, a commercially available invasion assay (Cell Biolabs, Inc.) was used according to the manufacturers' instruction. Briefly, the membrane was rehydrated, and cell culture media containing 10% FBS (vehicle) or 10% FBS with BAFF (150 ng/ml) or 10% FBS with APRIL (150 ng/ml) was added to the lower well of the invasion plate. Subsequently, cells in single-cell suspension were placed in the insert. After 48 h incubation at 37°C, the invasive cells were stained with cell stain solution, lysed with extraction solution and the absorbance was measured at

560 nm using a plate reader (Ledetect; Labexim Products). The data were analyzed using MicroWin2000 software (OEM version; Labexim Products).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc.). Data were compared using Mann-Whitney U test or Kruskal-Wallis followed by Dunn's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. The results from at least five independent experiments are presented as the median \pm interquartile range.

Results

NSCLC cells express BAFF, APRIL and their receptors. First, we aimed to investigate whether NSCLC cells could express BAFF, APRIL and their receptors BCMA, TACI and BAFF-R using RT-qPCR and western blotting (Fig. 1). The results demonstrated that A549 and H2030 cell lines expressed BAFF, APRIL and BAFF-R (Fig. 1A-C). Conversely, the expression

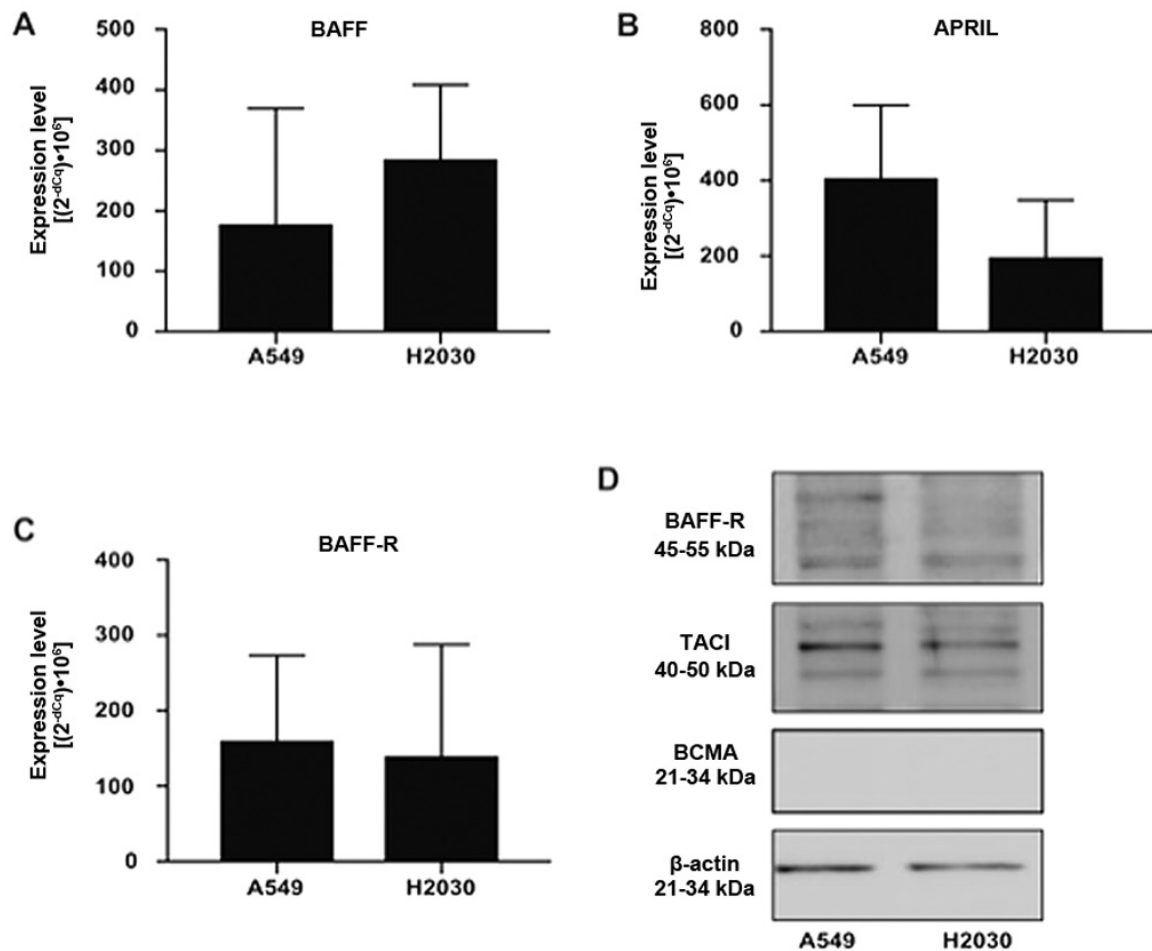


Figure 1. BAFF-R and TACI are expressed in non-small cell lung cancer cells. (A) BAFF, (B) APRIL and (C) BAFF-R gene expression levels in A549 and H2030 cell lines. (D) Representative western blottings for BAFF-R, TACI and BCMA in A549 and H2030 cell lines. No significant differences were observed. APRIL, a proliferation-inducing ligand; BAFF-R, B-cell activating factor receptor; TACI, transmembrane activator and CAML interactor; BCMA, B-cell maturation antigen.

of TACI and BCMA was detected in H2030 cells only, while in the A549 cell line, the expression of both receptors was under the detection limit (data not shown). Furthermore, no difference was found in the expression levels of BAFF, APRIL and BAFF-R among the analyzed cell lines (Fig. 1A-C). In addition, results from western blotting demonstrated that both cell lines expressed BAFF-R and TACI at the protein level, while no expression of BCMA was detected (Fig. 1D). These findings suggested that NSCLC cells could respond directly to BAFF and APRIL stimulation via BAFF-R and TACI signaling.

Effect of different BAFF and APRIL doses on NSCLC cells viability. Having found that both cell lines possess the potential to directly respond to BAFF and APRIL stimulation by the interaction with TACI and BAFF-R, we subsequently aimed to investigate the direct effects of both ligands on cancer cell viability. As expected, the results demonstrated that stimulation of H2030 and A549 cells with 50, 100 and 150 ng/ml BAFF (Fig. 2A and B) and APRIL (Fig. 2C and D) did not affect NSCLC cell viability. These findings indicated that BAFF and APRIL were not toxic for NSCLC cells and could be used in further functional experiments.

NSCLC-derived BAFF and APRIL do not influence cell proliferation. Having found that both cell lines can produce the analyzed ligands, we aimed to investigate whether A549 and H2030 cell-derived BAFF and APRIL may directly influence their proliferation (Fig. 3). Functional experiments were performed with receptor blocking using functional-grade monoclonal antibodies. The results demonstrated that blocking TACI and BAFF-R separately or in combination did not influence the proliferation of A549 (Fig. 3C) and H2030 (Fig. 3D) cells. These results indicated that endogenous BAFF and APRIL may not increase tumor growth directly by increasing cancer cell proliferation.

Exogenous BAFF and APRIL did not influence NSCLC cell proliferation. Next, we wished to analyze whether exogenous BAFF and APRIL could directly affect NSCLC cell proliferation. Cells were stimulated with recombinant human BAFF and APRIL at 50, 100 and 150 ng/ml. The results demonstrated no effect of exogenous stimulation with both ligands on A549 (Fig. 4A) and H2030 (Fig. 4B) cell proliferation, since no differences in the frequency of proliferating cells was observed (Fig. 4C-F). Furthermore, no effect on cancer cell invasiveness in response to BAFF and APRIL stimulation was observed compared with vehicle alone (Fig. 5).

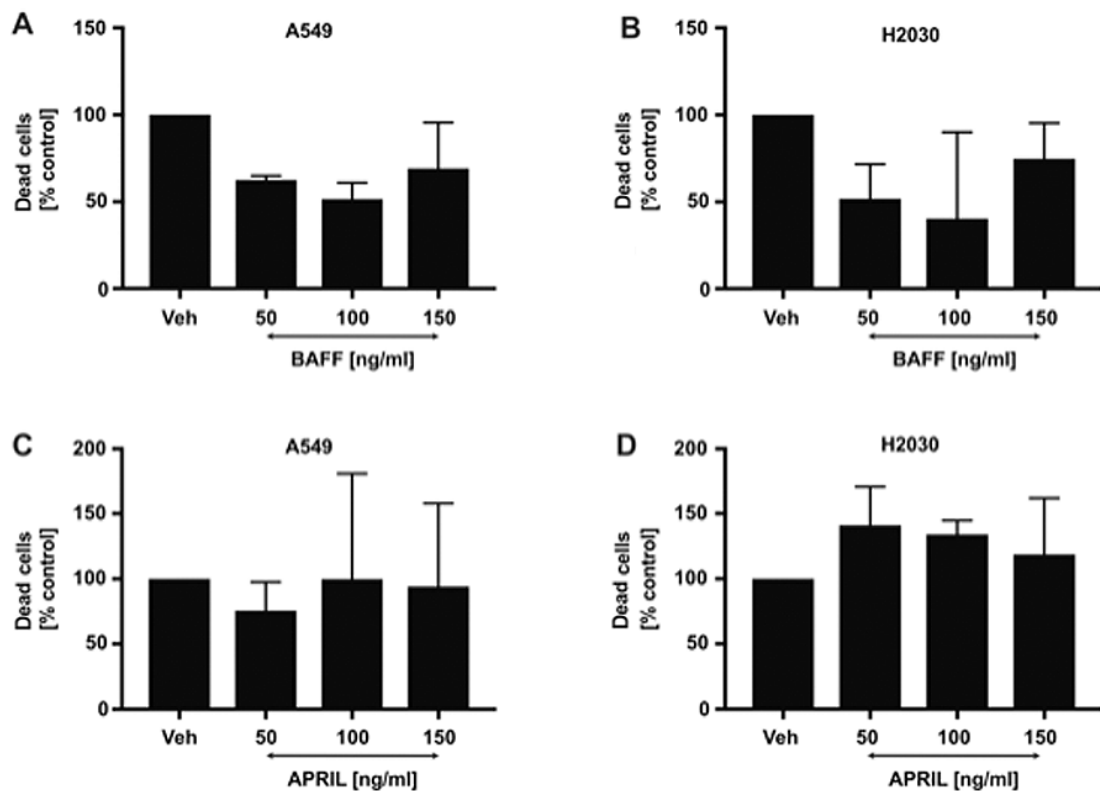


Figure 2. Effects of BAFF and APRIL signaling on non-small cell lung cancer cell viability. Effect of BAFF signaling on (A) A549 and (B) H2030 cell viability. Effects of APRIL signaling on (C) A549 and (D) H2030 cell viability. Cells were cultured in the presence of different concentrations of BAFF and APRIL or untreated (veh alone). No significant differences were observed. APRIL, a proliferation-inducing ligand; BAFF, B-cell activating factor; veh, vehicle.

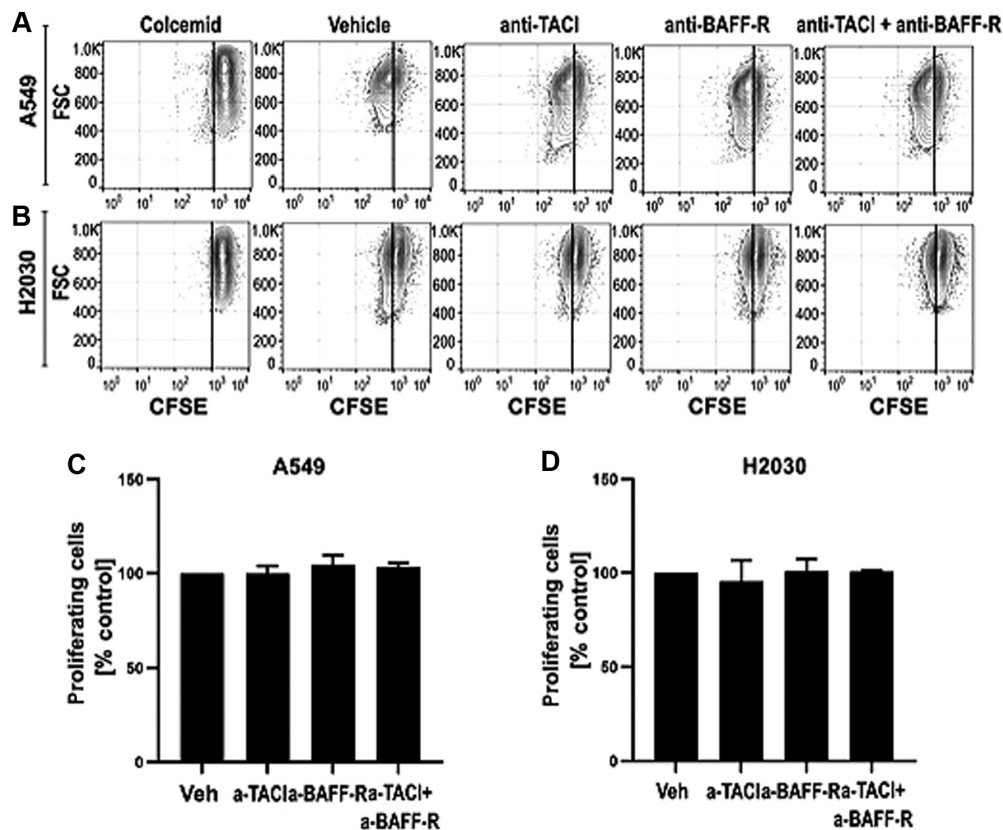


Figure 3. Endogenous BAFF and APRIL do not change non-small cell lung cancer cell proliferation. Representative flow cytometry density plots for CFSE based proliferation of (A) A549 and (B) H2030 cell lines with blocked TACI (anti-TACI) and/or BAFF-R (anti-BAFF-R). Effects of blocking TACI and/or BAFF-R in (C) A549 and (D) H2030 cell lines. Colcemid was used as a negative control of cancer cell proliferation (for gate set up). No significant differences were observed. BAFF-R, B-cell activating factor receptor; TACI, transmembrane activator and CAML interactor; vh, vehicle

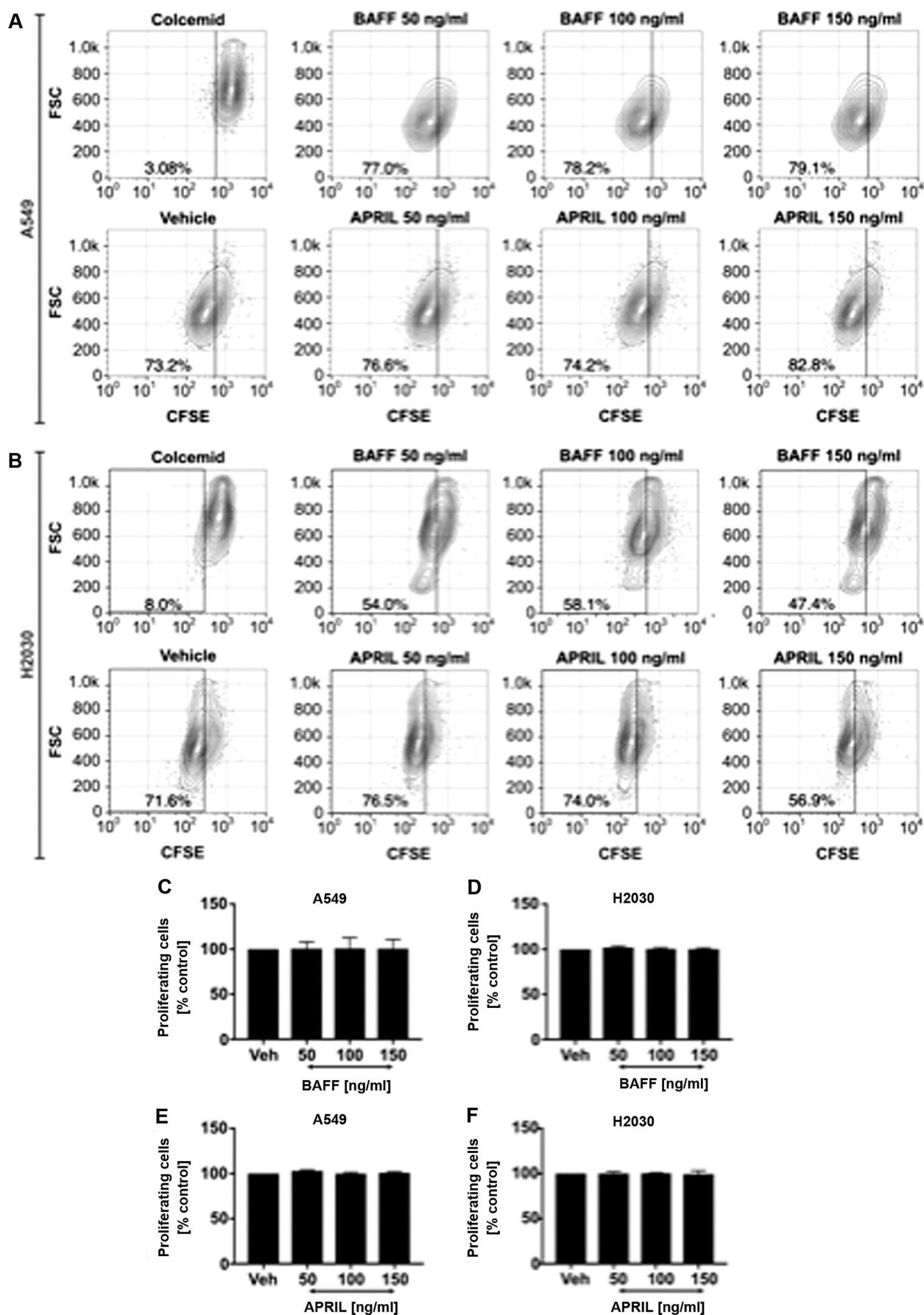


Figure 4. Direct effects of exogenous BAFF and APRIL signaling on non-small cell lung cancer cell proliferation. Representative flow cytometry density plots for CFSE based proliferation in BAFF and APRIL-treated (A) A549 and (B) H2030 cells. Effects of different concentrations BAFF and APRIL on A549 (C and E, respectively) and H2030 (D and F, respectively) cells. Colcemid was used as a negative control of cancer cell proliferation (for gate set up). No significant differences were observed. APRIL, a proliferation-inducing ligand; BAFF, B-cell activating factor; veh, vehicle.

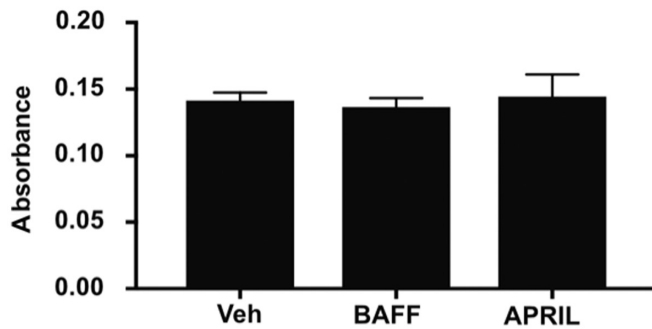


Figure 5. The influence of BAFF and APRIL signaling on non-small cell lung cancer cell aggressiveness. Effect of 150 ng/ml BAFF and APRIL treatments on A549 and H2030 cancer cell aggressiveness. 10% FBS (vehicle) was used as a control. No significant differences were observed. APRIL, a proliferation-inducing ligand; BAFF, B-cell activating factor; veh, vehicle.

Discussion

The present study demonstrated that NSCLC cells possess the ability to respond directly to BAFF and APRIL stimulation via interaction with TACI and BAFF-R, while BCMA was undetectable at the protein level. Previous studies reported that BAFF and APRIL can enhance the proliferation of normal and malignant B cells (11,20). In addition, previous studies from our group demonstrated that the expression of BCMA might serve as a prognostic factor for treatment response in patients with acute myeloid leukemia (8,9). In solid tumors, BAFF and APRIL signaling is associated with cancer cell proliferation, such as in breast cancer (12), esophageal cancer (26), clear cell renal cell carcinoma (27), adult male germ cell tumor (28) and invasive bladder carcinoma (29). Furthermore, elevated local and systemic levels of BAFF and APRIL were shown to be associated with a higher stage and disease progression, such as in chronic graft versus host disease, systemic lupus erythromatosus and breast cancer tumor size (12,13). Surprisingly, the present study demonstrated that BAFF and APRIL direct signaling did not serve an essential role in NSCLC aggressiveness, as no effect of BAFF and APRIL on NSCLC cell proliferation and invasiveness was reported. Conversely, previous studies reported that APRIL signaling in NSCLC cells promote tumor proliferation, migration and metastasis (23,30). Consequently, both ligands may act as tumor supporters and pro-metastatic factors in an indirect manner. In fact, high BAFF and APRIL expression were shown in NSCLC tissues (22). It is therefore essential to determine whether BAFF and APRIL signaling in tumor-infiltrating or stromal cells, including tumor-infiltrating macrophages or tumor-associated fibroblasts, may play a role in tumor progression and spread.

Macrophages (alternatively activated) and fibroblasts are crucial components of tumor stroma. The progressive effects of macrophages and fibroblasts on tumor growth are associated with the release of growth factors and anti-inflammatory mediators, their promotion of cancer invasiveness by releasing matrix degradation enzymes, and their involvement in the recruitment of suppressive cells, including T regulatory cells to the tumor side (31,32). Both aforementioned cell subsets have been shown to produce high levels of BAFF and APRIL; however, the effects of both ligands on the activation and biological function of tumor-infiltrating macrophages and

tumor-associated fibroblasts remain elusive (18,24,33-35). BAFF via direct interaction with TACI may therefore induce monocyte maturation towards macrophages and, thus, contribute to tumor progression.

BAFF and APRIL signaling stimulate the expression of anti-apoptotic proteins, such as Bcl-2, Bcl-XL and Bcl-2-related protein A1 (36,37). Both ligands may therefore serve as anti-apoptotic mediators and increase the survival of cancer cells subjected to chemotherapy (11). Interestingly, APRIL gene silencing was shown to increase the apoptotic susceptibility of gastric cancer cells (38). However, the present study demonstrated that BAFF and APRIL stimulation had no effects on the viability of NSCLC cells in normal conditions. Further investigation is therefore required to elucidate the impact of BAFF and APRIL on cancer cell susceptibility to cytotoxic therapy.

In summary, the results from the present study demonstrated that, despite the presence of TACI and BAFF-R in NSCLC cells, both BAFF and APRIL did not exert direct effects on cancer cell proliferation and invasiveness. Further studies are needed to elucidate the mechanisms of previously reported *ex vivo* associations between BAFF and APRIL and cancer progression (12,13).

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AE, MM and LB developed the research concept. AE and LB designed the experiments. MW, MT, DL and KG performed the experiments. MW, MT and JD performed statistical analysis and designed figures. MW and MT drafted the manuscript. AE, LB, JD and MM revised the draft. AE, MM and BL confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Patient consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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