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Enhanced angiogenesis of human umbilical vein endothelial cells via THP-1-derived M2c-like macrophages and treatment with proteasome inhibitors 'bortezomib and ixazomib'

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The leading cause of cancer-related death is lung cancer, with metastasis being the most common cause of death. To elucidate the role of macrophages in lung cancer and angiogenesis processes, we established an in vitro co-culture model of A549 or HUVEC with THP-1 cells that polarized to M2c macrophages with hydrocortisone. The proteasome inhibitors bortezomib and ixazomib were investigated for their effects on proliferation, invasion, migration, metastasis, and angiogenesis pathways. The effects of bortezomib and ixazomib on gene expression in gene panels, including crucial genes related to angiogenesis and proteasomes, were investigated after the co-culture model to determine these effects at the molecular level. In conclusion, bortezomib and ixazomib showed antiproliferative effects in both cells, as well as in M2c macrophage co-culture. M2c macrophages also increased invasion in A549 cells and both invasion and migration in HUVEC. mRNA expression upregulation, specifically in the NFKB and VEGF genes, supported the metastatic and angiogenic effects found in A549 and HUVEC with M2c macrophage co-culture. Additionally, bortezomib inhibited the VEGFB pathway in HUVEC and NFKB1 in A549 cells. The significant findings obtained as a result of this study will provide information regarding angiogenesis induced by M2 macrophages.

Key words: HUVEC; macrophage; hydrocortisone; bortezomib; ixazomib.

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INTRODUCTION

Lung cancer is the most common type of cancer and the main cause of cancer-related death worldwide [1]. Currently, the primary goals of lung cancer research are to evaluate the molecular pathways of lung cancer, especially the unique mechanisms of its ability to metastasize, and to evaluate the associated therapeutic treatment scientifically and effectively [2]. Examining molecular pathways and treatments alongside other cells in the tumor microenvironment is one of the most remarkable problems in cancer research today. Tumor-associated macrophages (TAMS) play a crucial role in the development of cancer progression, angiogenesis, cancer metastasis, and drug resistance [3–6]. The microenvironment identifies non-cancerous cells and structures within the tumor tissue, and approximately 40% of the mass of solid tumors consists of TAMs [6, 7]. Macrophages are one of the most important members of the tumor microenvironment and can express pro-tumoral and anti-tumoral functions. TAMs' tumor-promoting activities are mediated by functional mechanisms such as cell proliferation and

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invasion, destruction of adaptive immunity, extracellular matrix remodeling, and angiogenesis [8–10]. Plasticity is a distinguishing feature of cells of monocyte–macrophage origin, and macrophages have a high level of cellular plasticity. These cells differentiate into proinflammatory type I macrophage (M1) or anti-inflammatory type II macrophage (M2) phenotypes in response to various agents [11]. Macrophages at rest (M0 macrophages) are polarized into M1 macrophages with stimuli such as interferon-gamma (IFN-γ) or lipopolysaccharide (LPS) alone or cytokines such as tumor necrosis factor-alpha (TNF-α). Macrophages in M2 form are also called anti-inflammatory or regulatory subgroups. M2 macrophage cells facilitate the processes of healing wounds, angiogenesis, tissue remodeling, and the development of tumors [12–14]. M2 macrophages are polarized by different stimuli into alternatively activated macrophage types 2a, 2b, and 2c (M2a, M2b, and M2c). M2 macrophages are polarized into the M2c subtype by interleukin-10 (IL-10), transforming growth factor-beta (TGF-β), and glucocorticoids [3, 7]. In a previous study, we used hydrocortisone, a 'well-known glucocorticoid', for the first time in M2c polarization, with a particular emphasis on M2c macrophages [3]. Corticosteroids are synthetic analogues of naturally occurring corticosteroid hormones that are used clinically in many lung-related diseases, such as asthma, pneumonia, and coronavirus disease 2019 (COVID-19) [15]. As a result, studies of metastasis and angiogenesis mechanisms become important in the co-culture model we developed using hydrocortisone in M2c polarization. Proteasome inhibitors have a unique mechanism of action. Bortezomib is the first drug from this group to reach the clinical trial period and be approved by the Food and Drug Administration (FDA). Bortezomib is clinically used in the treatment of multiple myeloma and mantle cell lymphoma [16], and its effects on many solid tumors are still being studied [3, 17]. Although bortezomib has been used to treat cancers of hematopoietic origin, such as myeloma, growing basic and clinical studies have also shown that bortezomib is successful in treating many types of solid tumors [18–20]. Ixazomib is a boron-containing small peptide inhibitor molecule, a second-generation proteasome inhibitor developed to treat many tumors. Unlike bortezomib, ixazomib has oral bioavailability and greater tissue distribution suitability [21]. In our study, we used an *in vitro* human monocyte cell (THP-1)-derived M2c-polarized macrophage cell model formed with hydrocortisone. In this study, the antimetastatic effects of proteasome inhibitors bortezomib and ixazomib, which are currently used in cancer therapy, were determined in non-small cell lung cancer (A549) co-cultures with M2c-polarized macrophages, as well as the effects on anti-angiogenic mechanisms in human umbilical vein endothelial cells (HUVEC).

MATERIALS AND METHODS

Chemicals

All chemicals and reagents were of analytical grade. RPMI-1640, fetal bovine serum (FBS), phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), phorbol 12-myristate 13-acetate (PMA), penicillin–streptomycin, and hydrocortisone were purchased from Sigma Aldrich (St. Louis, MO, USA). Bortezomib was purchased from BioChemica (Sauerlach, Germany), and ixazomib was purchased from Active Biochem (Germany). CD-206 (Cat no. 321104) and CD163 (Cat no. 326506) were purchased from BioLegend (San Diego, CA, USA). BD Biosciences (ABD) supplied the Matrigel.

Cell culture and treatments

Human umbilical vein endothelial cells (American Type Culture Collection (ATCC), CRL-1730™), A549 non-small cell lung cancer cells (CCL-185, ATCC®, USA), and THP-1 human monocyte cells (TIB-202, ATCC®, USA) were cultured in RPMI-1640 medium with 10% FBS and 1% penicillin–streptomycin. All cells were cultured in a Heracell™ CO_2 incubator (Thermo Fisher Scientific™, Pittsburgh, PA, USA) at 37 °C, 5% CO_2, and 95% humidity. Bortezomib and ixazomib were dissolved in DMSO and diluted to the required concentrations with fresh medium. Prior to the experimental procedures, cellular samples were subjected to staining using a trypan blue solution and subsequently assessed using a cell counting device (Cedex, Roche). The experiments were carried out utilizing viable cells in appropriate quantities.

Macrophage polarization and co-culture studies

Our previous study described in detail the polarization of THP-1 monocytes into M2c macrophages [3]. Briefly, in order to induce M0 macrophage polarization, THP-1 cells were incubated in 6-well plates with 100 ng/mL PMA for 24 h. 1 μM hydrocortisone was applied for 72 h of incubation to differentiate M0 macrophages into M2c-polarized macrophages. In order to verify the M2c polarization, the flow cytometry (BD Accury C6) method was used to measure the expression levels of CD-206 and CD163, which are recognized as cell surface markers for M2 and M2c-polarized macrophages, respectively. To provide a suitable environment for the coexistence of HUVEC or A549 cells and M2 macrophages, co-culture plates were employed (Corning, NY, USA). The HUVEC or A549 cells were co-cultured with M2c macrophages in six-well plate (or for RTCA methods, e-plate insert) cell culture inserts with a 0.4 μm porous membrane separating the upper and lower chambers, allowing for the exchange of soluble factors but not cell transmigration. To ensure consistency between experiments, THP-1 cells were plated in the upper chamber at a 5:1 ratio to the number of

HUVEC or A549 cells plated in the lower chamber. The two cell populations were incubated in the presence of IC_{50} concentrations of bortezomib or ixazomib added directly into the wells for 24 h.

Proliferation assay by real-time cell analysis (RTCA)

The A549 and HUVEC were prepared at a concentration of 1×10^4 cells/well. About 200 μL of medium was added to the wells of an E-Plate 16 (Agilent, CA, USA), and the E-Plate 16 was then placed on the RTCA station for baseline measurements. After 24 h of incubation, the instrument was paused, and 100 μL of the medium was removed from the wells, and bortezomib and ixazomib were added at various concentrations (1, 100 nM, and 1, 10 μM). The sample was positioned on an RTCA station within the incubator for an extended period to quantify cellular proliferation. Cell proliferation and IC_{50} concentrations of bortezomib and ixazomib were monitored and evaluated for 24 h according to the cell index values by RTCA DP Software (Agilent, version numbers 1.2.1) [22].

Real-time monitoring of HUVEC proliferation under co-culture conditions with M2c-polarized macrophages using the E-plate insert

The RTCA system and RTCA E-Plate insert (Agilent, CA, USA) were used to measure, process, and analyze the impedance (cell index). The RTCA DP Software (Agilent, version numbers 1.2.1) was used to analyze the data. The E-plate 16 was filled with 160 μL HUVEC suspension (1×10^4 cells/well) with RPMI medium, and the plate was kept at room temperature for 30 min, until the suspension settled at the bottom of the plate. After incubation, cells were incubated for 24 h at 37 °C and 5% CO_2 in the RTCA cradle. Following a 24-h incubation period, culture medium was removed and replaced with 80 μL of serum-free medium containing bortezomib and ixazomib IC_{50} concentrations. At the same time, E-Plate inserts, which contain serum-free medium and M2c-polarized macrophages, were lowered into the E-16 plate wells for cell stimulation. The control consisted of a well with no cells seeded in the same insert(s). The impedance recording was then resumed on the machine for a duration of 24 h at 1-h intervals. Four wells were used for each treatment group in a 16-well plate assay, and the average of the four wells was used as the cell growth index of the treatment. Cell growth (measured as cell index) was monitored continuously for a day. Each independent experiment was performed in triplicate [3].

Determination of antimetastatic and anti-angiogenic effects in co-culture with M2c-polarized macrophages

Invasion and migration studies were performed on A549 lung cancer cells for the antimetastatic effect and HUVEC for the anti-angiogenic effect in the RTCA-DP system (Agilent, CA, USA). Cell invasion and migration were evaluated using the RTCA system as per the manufacturer's guidelines. Briefly, a 5% matrigel was made with serum-free medium for the invasion study. The upper chamber of the CIM plate (Agilent, CA, USA) was loaded with 20 μL of matrigel, which was then incubated for 4 h at 37 °C. After the incubation period, the upper and lower chambers of the CIM plate were merged, and medium containing serum was added to the lower chambers for the invasion and migration studies. In total, 80 μL of serum-free media was used to seed A549 or HUVEC (2×10^4 cells/well) onto the top chambers of CIM-16 plates. In total, 100 μL of bortezomib or ixazomib IC_{50} concentrations was then added, and the RTCA DP system was used to collect data. At least once every 10 min, the RTCA DP software was configured to collect impendence data [3].

RNA extraction and real-time PCR

The MagNA Pure Compact RNA Isolation Kit (Roche, Lot: 13243700) and the MagNA Pure Compact Instrument LC 2.0 system (Roche Diagnostics, Mannheim, Germany) were used to lyse cells for the extraction of total ribonucleic acid (RNA), and the High-Fidelity cDNA Synthesis Kit (Roche, Germany) was used for the reverse transcription of RNA into complementary DNA (cDNA). The high quality of the RNA samples was confirmed by using the NanoDrop Instrument at 260 and 280 nm optical densities. From each RNA population, 100 ng of total RNA was used for cDNA synthesis with the Transcriptor High Fidelity cDNA Synthesis Kit (Cat no. 05091284001, Roche), and reverse transcription reactions were performed according to the kit procedure. The quality of the cDNA samples was also confirmed with the NanoDrop instrument. The LightCycler® 480 Probes Master (Cat no. 04 707 494 001) Kit (Roche Applied Science, Germany) was used for quantitative fluorescence determination. According to the instructions, sample mixes were added to a 96-well plate, which was then placed in the LightCycler® 480 instrument (Roche Applied Science, Germany) to initiate the reaction [23, 24]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The sequences of the primers used are presented in Table 1.

Statistical analysis

The statistical analysis of the results was performed using one-way ANOVA and Tukey's post hoc test with GraphPad Prism 6.0 software. The results are the means of three independent experiments expressed, as the mean ± standard deviation. p values represent the significance of the results compared to the control group ($p > 0.05$ n.s., $p < 0.05$*, $p < 0.01$**, $p < 0.001$***, and $p < 0.0001$****).

RESULTS AND DISCUSSION

M2c macrophage polarization

The expressions of M2 and M2c markers in differentiated macrophage subtypes were measured using flow cytometry to confirm M2c polarization. As a result, the M2c macrophages were characterized by CD206 (13.7%) and CD163 (10.8%) membrane receptor levels by flow cytometry (Fig. 1).

M2 macrophages, also known as alternatively activated macrophages, have been observed to exhibit a functionally distinct phenotype compared

Table 1. Gene symbols and primer sequences

Gene symbol	Gene	Forward primer- reverse primer sequence
NFk-B1	Nuclear factor kappa B subunit 1, NF-kB-p50 (NF-kB-p105)	Forward: 5′- CTGGCAGCTCTTCTCAAAGC -3′ Reverse: 5′- TCCAGGTCATAGAGAGGCTCA -3′
NFk-B2	Nuclear factor kappa B subunit 2 (p52)	Forward: 5′- CCCATCCATGACAGCAAAT -3′ Reverse: 5′- CTTGTCACAAAGCAGATAAACTTCA -3′
NFk-BIA	NF-kB inhibitor alpha (IkBA)	Forward: 5′- GCTGATGTCAATGCTCAGGA -3′ Reverse: 5′- ACACCAGGTCAGGATTTTGC -3′
NFk-BIB	NF-kB inhibitor beta (IkBB)	Forward: 5′- AGAGTGAGGAGGACTGGAAGC -3′ Reverse: 5′- CTCCACATCTTTGTGGATAACG -3′
TP53	Tumor protein p53	Forward: 5′- AGGCCTTGGAACTCAAGGAT -3′ Reverse: 5′- CCCTTTTTGGACTTCAGGTG -3′
EGFR	Epidermal growth factor receptor	Forward: 5′- CAGCCACCCATATGTACCATC -3′ Reverse: 5′- AACTTTGGGCGACTATCTGC -3′
EGF	Epidermal growth factor	Forward: 5′- CCTCAGATGGGAAAACGTG -3′ Reverse: 5′- GTTCTTTAGATCAACTTCACCACCT -3′
COX10	Cytochrome c oxidase assembly protein	Forward: 5′- CGTAAATTATGGCCGCATCT -3′ Reverse: 5′- GACAGAGCCTCCTACGCAAC -3′
VEGFA	Vascular endothelial growth factor A	Forward: 5′- GCAAGACAAGAAAAATGTGACAA -3′ Reverse: 5′- TGGTTTCTGTATCGATCGTTCT -3′
VEGFB	Vascular endothelial growth factor B	Forward: 5′- CTGGCCACCAGAGGAAAGT -3′ Reverse: 5′- CATGAGCTCCACAGTCAAGG -3′
MMP9	Matrix metallopeptidase 9	Forward: 5′- ATCCGGCACCTCTATGGTC -3′ Reverse: 5′- CAGACCGTCGGGGGAG -3′
MMP17	Matrix metallopeptidase 17	Forward: 5′- CGACCACAAGATCGTCTTCTTT -3′ Reverse: 5′- CGGGTATCCTTCCTCTACGTT -3′
ANGPT1	Angiopoietin 1	Forward: 5′- AGCTACCACCAACAACAGTG -3′ Reverse: 5′- CAAAGATTGACAAGGTTGTGG -3′
ANGPT2	Angiopoietin 2	Forward: 5′- GGATGGAGACAACGACAAATG -3′ Reverse: 5′- GGACCACATGCATCAAACC -3′
IL-6	Interleukin 6	Forward: 5′- CCAGAGCTGTGCAGATGAGT -3′ Reverse: 5′- GGGTCAGGGGTGGTTATTG -3′
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Forward: 5′- CTCTGCTCCTCCTGTTCGAC -3′ Reverse: 5′- ACGACCAAATCCGTTGACTC -3′

to M1 macrophages. M2 macrophages undergo polarization in response to various stimuli, resulting in their differentiation into distinct subtypes known as M2a, M2b, and M2c macrophages [25, 26]. Our study's co-culture method uses M2c macrophages, which become active in response to conditions with high levels of IL-10, TGF-β, glucocorticoids, or immune complexes [27]. The surface marker profile of M2 macrophages is characterized by the presence of CD23, the scavenger receptors CD163 and CD204, and the mannose receptor CD206. Furthermore, the upregulation of CD163 is a well-established indicator of M2c polarization, as demonstrated by Sousa et al. in 2015 [7]. In our previous study, we conducted comprehensive optimization scans of M2c macrophage polarization [3]. The observed elevation of CD206 and CD162 levels in this study compared to the control group indicated the presence of M2c polarization.

Effects of proteasome inhibitors on cell viability

The results of bortezomib and ixazomib concentrations (1, 100 nM and 1, 10 μM) on HUVEC and A549 cell viability as determined by real-time recorded data on the RTCA system at 24 h are presented in Figs. 2 and 3. Using RTCA DP cell index values, the IC_{50} concentrations for bortezomib and ixazomib in A549 and HUVEC were calculated with the RTCA DP Software 1.2.1 program. Bortezomib's IC_{50} concentrations were calculated as 172.48 and 101.65 nM for A549 and HUVEC, respectively. Also, ixazomib's IC_{50} concentrations were calculated as 2.198 μM and 77.802 nM for A549 and HUVEC, respectively. These results suggest that decreases in cell viability measured by a real-time cell analysis assay were observed in a dose-dependent manner compared to the control.

In our previous study, a real-time cell analysis system was used to determine the 48-h IC_{50} concentrations of the drugs bortezomib and ixazomib in A549 cells [3]. Additionally, IC_{50} concentrations have been determined using various methods, including MTT with A549 cells and bortezomib, in numerous studies [28–31]. Likewise, there are current studies in the literature that examine the effects of bortezomib on HUVEC viability, which correlate with our findings [32–34]. The effects of ixazomib

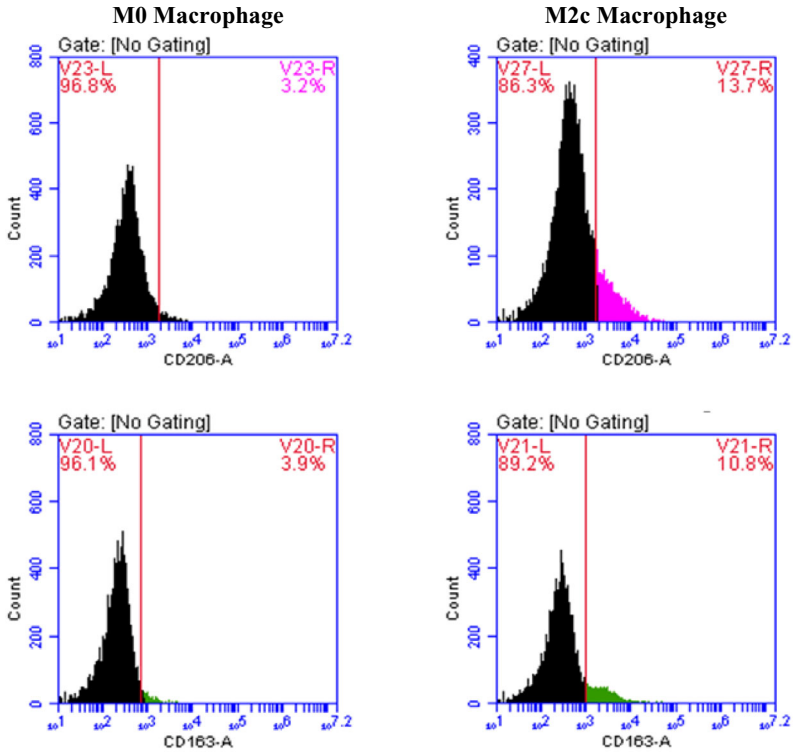

Fig. 1. Validation of M2c polarization by flow cytometry. CD163 are specific surface markers of M2c macrophages, and the marker CD206 is common to M2 subtypes. Experiments were performed in triplicate independently; at least 10 000 cells were analyzed per sample (%).

on cell viability in HUVEC have not been extensively studied in the scientific literature. As a result, the real-time cell analysis system used to determine the ixazomib IC_{50} concentration in HUVEC has made a significant contribution to the literature.

Cell proliferation analysis in m2c-polarized macrophages and HUVEC co-culture in RTCA

In M2c + HUVEC co-culture groups, the direct effects of M2c-polarized macrophages on HUVEC proliferation were analyzed. In our investigation, we improved the accuracy of our findings by using a co-culture system including HUVEC together with M2c-polarized macrophage cells. The use of E-plate inserts, which allowed simultaneous growth in the same culture medium, assisted in this co-culture. Additionally, we used real-time methods of measurement to monitor the proliferation of these cells. The results were compared with the HUVEC control group. There was no significant difference in the proliferation curves of the M2c + HUVEC co-culture group compared to the HUVEC control group. Furthermore, when HUVEC + M2c macrophage co-culture groups were incubated for 24 h with bortezomib and ixazomib IC_{50} concentrations, HUVEC proliferation was significantly reduced compared to the HUVEC control group (Fig. 4).

In our previous investigation, we assessed the effect of M2c-polarized macrophages on the proliferation of A549 cells using real-time cell host data collected over a 48-h period [3]. Research has demonstrated that interacting with TAMS can modulate the proliferation of malignant cells and influence their responsiveness to chemotherapeutic agents. Various subtypes of macrophages, including M2c, present in the periphery of tumors have been shown to exert distinct effects on cell proliferation [35].

Antimetastatic activity in co-culture with M2c-polarized macrophages and A549 lung cancer cells

Antimetastatic activities were investigated using invasion and migration studies on A549 lung cancer cells. As a result, the invasion ability of A549 lung cancer cells co-cultured with M2c-polarized macrophages was statistically significantly increased ($p < 0.05$*) when compared to the A549 control group. The IC_{50} concentrations of bortezomib and ixazomib significantly inhibited the invasive effect, which increased in A549 lung cancer cells in the

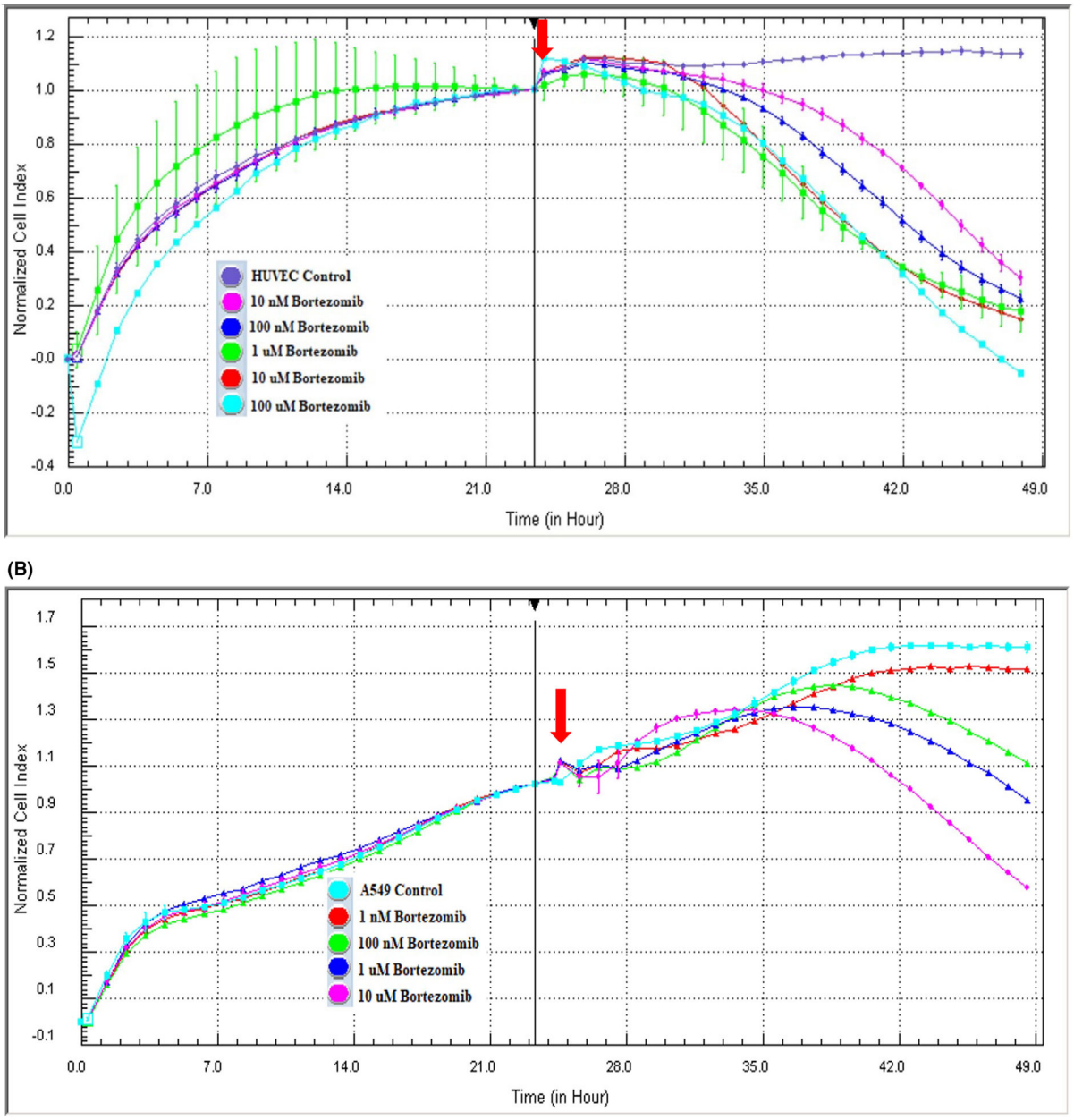

Fig. 2. Antiproliferative effects of bortezomib on HUVEC and A549 cells. (A) Real-time monitoring of the antiproliferative effects of bortezomib on HUVEC (IC_{50}: 101.65 nM) and (B) A549 cells (IC_{50}: 172.48 nM) at 24 h, results obtained using the RTCA DP system. IC_{50} values were calculated using RTCA DP Software 1.2.1, mean ± SD, n = 6 (Arrow: application of concentrations to wells).

presence of M2c-polarized macrophages. M2c-polarized macrophages inhibited the migration of A549 cancer cells. Furthermore, bortezomib and ixazomib IC_{50} concentrations statistically reduced A549 cell migration, similar to invasion results (Fig. 5).

Recent research indicates that M2c macrophages play a significant role in contributing to the development of tumors [36]. In addition, *in vitro* experiments demonstrated that M2c macrophages stimulated the invasion of lung tumor cells [34]. Ongoing efforts are being made to develop pharmaceutical agents that not only inhibit the proliferation of cancer cells but also demonstrate antimetastatic properties [37]. In this context, the

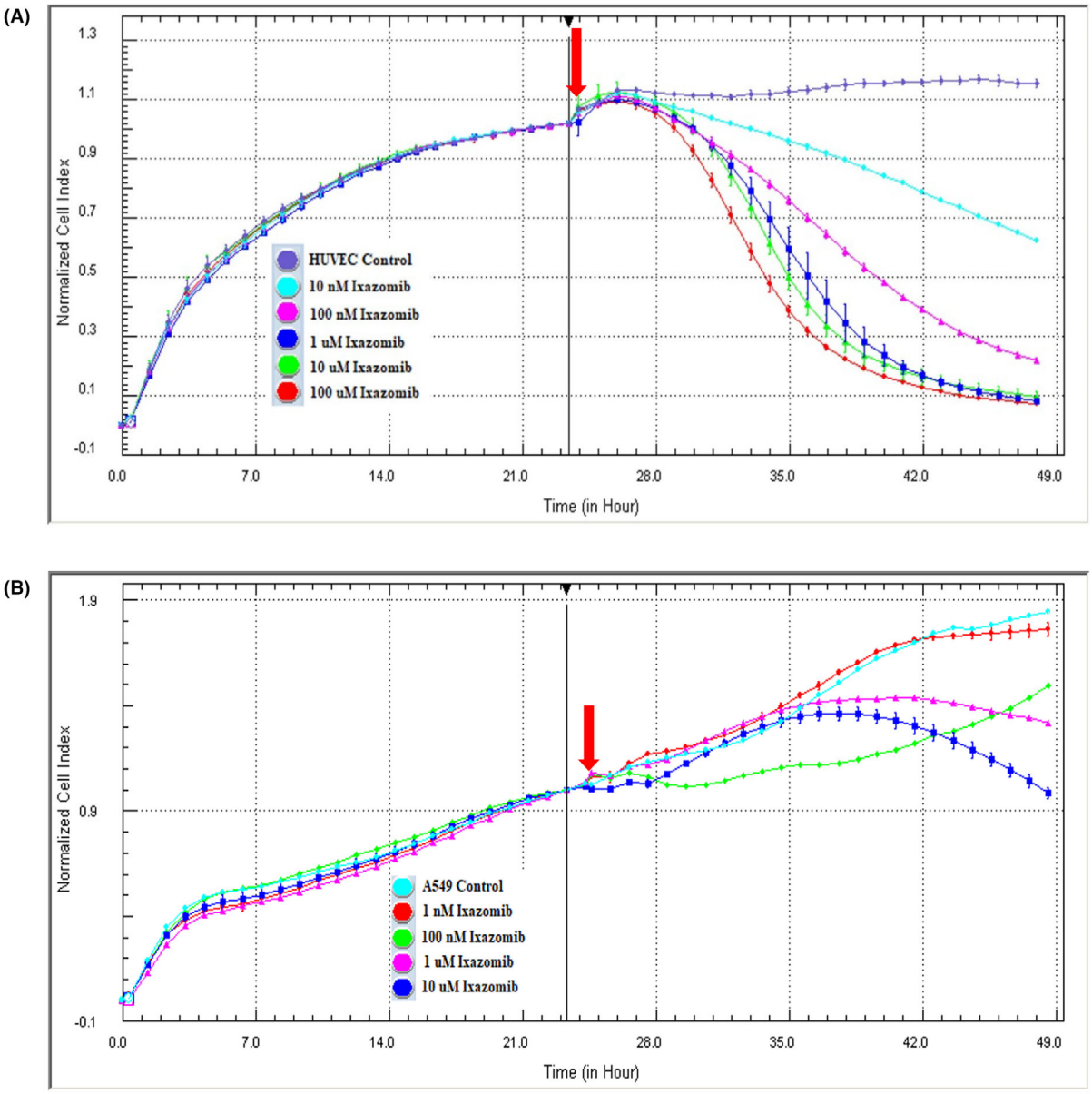

Fig. 3. Antiproliferative effects of ixazomib on HUVEC and A549 cells. (A) Real-time monitoring of the antiproliferative effects of ixazomib on HUVEC (IC_{50}: 77.802 nM) and (B) A549 cells (IC_{50}: 2.198 μM) at 24 h, results obtained using the RTCA DP system. IC_{50} values were calculated using RTCA DP Software 1.2.1, mean ± SD, n = 6 (Arrow: application of concentrations to wells).

inhibiting effects of bortezomib and ixazomib on increased invasion in the presence of M2c-polarized macrophages become significant.

Evaluation of anti-angiogenic effects in co-culture with M2c-polarized macrophages and HUVEC

Anti-angiogenic activities were investigated using invasion and migration studies on HUVEC with M2c macrophage co-cultures. This finding is important for the evaluation of the angiogenic effects of M2c-polarized macrophages on HUVEC. As a result, it was determined that both invasion and migration of HUVEC co-cultured with M2c-polarized macrophages were significantly increased compared to the control group. Although the angiogenic effect was higher in the presence of M2c-polarized macrophages, the IC_{50} concentrations of bortezomib and ixazomib significantly inhibited this effect (Fig. 6).

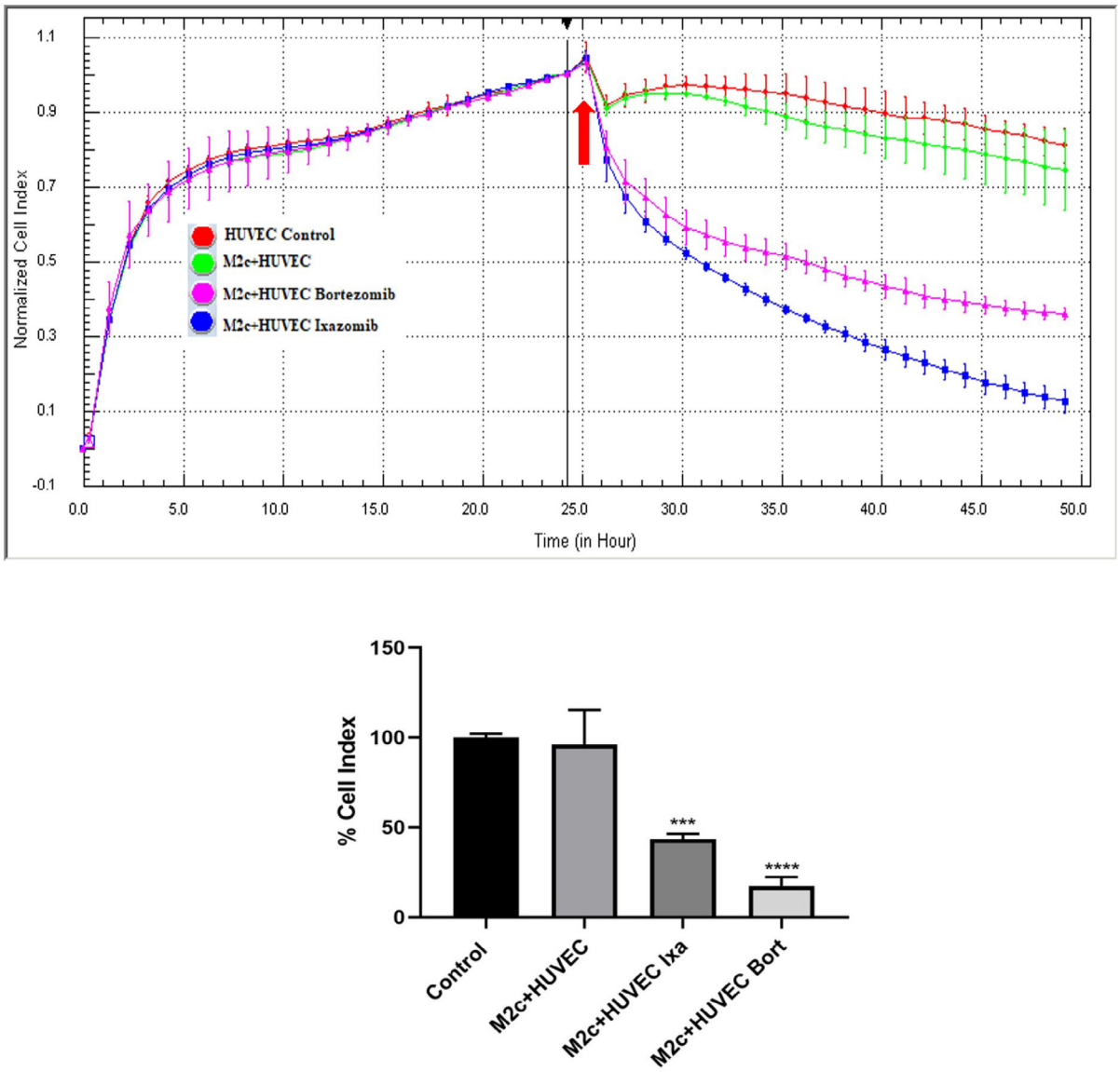

Fig. 4. Proliferation curves and column graph of co-culture models created with HUVEC and M2c-polarized macrophage cells using an E-plate insert, determined in a real-time cell analysis system (Arrow: merge of HUVEC and M2c macrophages, mean ± SD, n = 6, $p < 0.001$***, $p < 0.0001$****).

The process of angiogenesis is regulated by various factors, including secreted soluble molecules, low oxygen levels, components of the extracellular matrix (ECM), and direct cell–cell interactions with specific cell types, such as tissue-resident macrophages [38]. M2 macrophages facilitate ECM synthesis, cellular proliferation, and angiogenesis, as reported by Mantovani et al. (2004) and Martinez & Gordon (2014) [39, 40]. Specifically, M2c macrophages possess the capacity to modify the ECM [38, 41]. The findings suggest that macrophages that have undergone polarization toward an M2 phenotype exhibit an increased capacity for angiogenesis [42]. In summary, scientific research has demonstrated that M2c macrophages can stimulate the development of tumors by triggering the formation of new blood vessels, a process known as angiogenesis. This results from M2c macrophages promoting the migration and invasion of endothelial cells in both *in vitro* and *in vivo* studies [36, 42, 43].

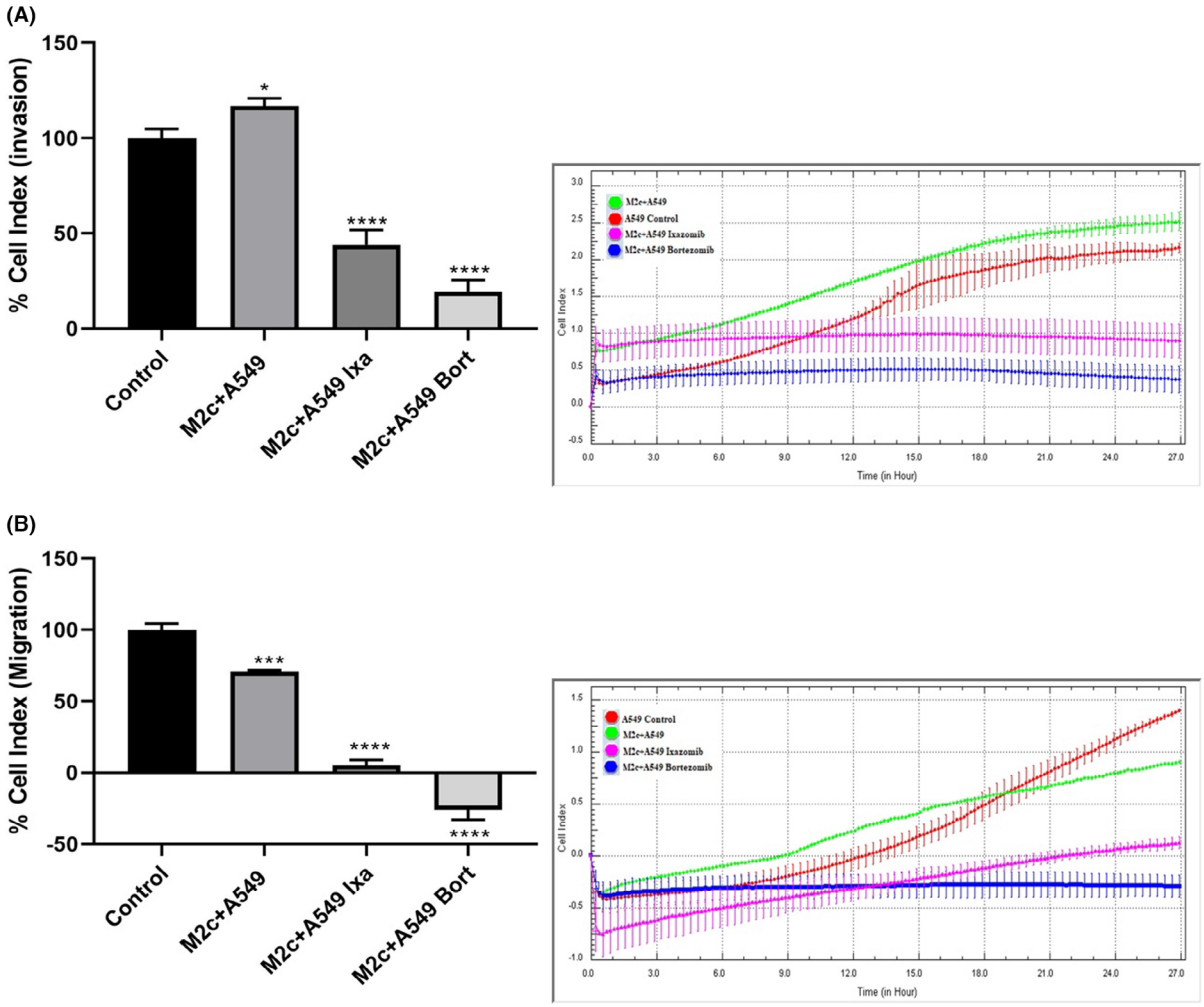

Fig. 5. Antimetastatic effect in co-culture with M2c-polarized macrophages and A549 cells. Anti-invasive (A) and anti-migration (B) effects of IC_{50} concentrations of bortezomib (IC_{50}: 172.48 nM) and ixazomib (IC_{50}:2.198 μM) on A549 cells (mean ± SD, n = 6, $p < 0.001$***, $p < 0.0001$****).

Evaluation of mRNA expression levels of angiogenesis and metastasis-related genes by real-time PCR

The expression levels of nuclear factor kappa b subunit 1 (NFk-B1), nuclear factor kappa b subunit 2 (NFk-B2), NF-kB Inhibitor alpha (NFk-BIA), NF-kB inhibitor beta (NFk-BIB), tumor protein 53 (TP53), epidermal growth factor receptor (EGFR), and cytochrome c oxidase assembly homolog 10 (COX10) genes in A549 lung cancer cells as associated with metastasis in the NF-kB family (Fig. 7A) and vascular endothelial growth factor a, b (VEGFA, VEGFB), matrix metalloproteinase-9, -17 (MMP9, MMP17), angiopoietin-1, -2 (ANGPT1, ANGPT2), COX10, epidermal growth factor (EGF), EGFR, and interleukin-6 (IL-6) gene mRNA expression levels selected as angiogenesis-related in HUVEC (Fig. 7B) were determined by the RT-PCR method. Gene panels were run in triplicate, and mRNA expression levels were normalized in target gene/reference gene ratios compared to the A549 or HUVEC control group. Based on the obtained findings, it has been determined that NFk-B1 ($p < 0.0001$****), NFk-B2 ($p < 0.05$*), NFk-BIA ($p < 0.01$**), NFk-BIB ($p < 0.001$***), and TP53 ($p < 0.0001$****) members of the NF-kB gene family exhibited increased levels in the M2c polarized macrophage co-culture group. These specific genes are known to be closely associated with cancer proliferation, metastasis, and particularly the proteasome pathways. Furthermore, there was a notable elevation in mRNA expression levels within the M2c macrophage group as compared to

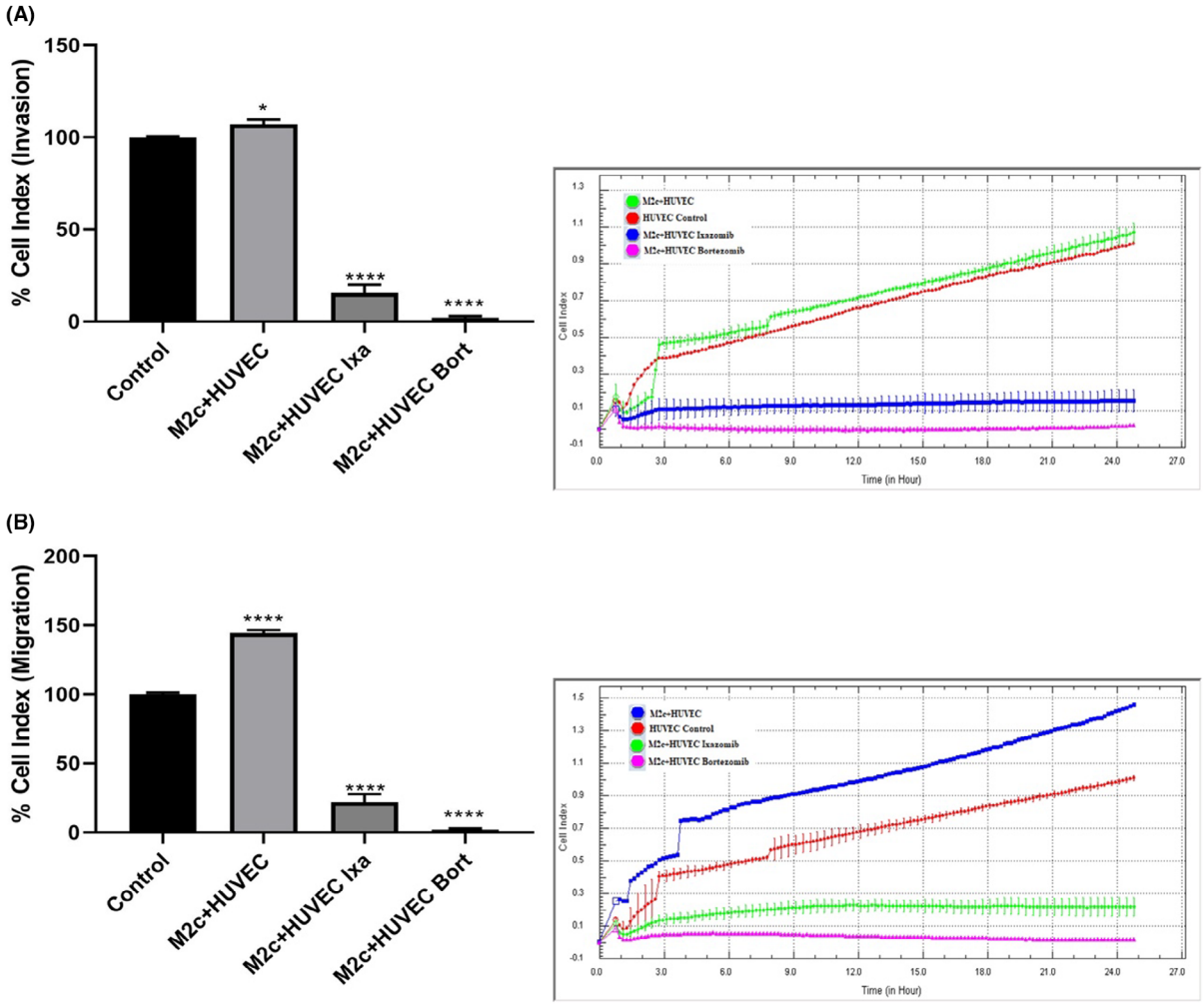

Fig. 6. Anti-angiogenic effect in co-culture with M2c polarized macrophages and HUVEC. Anti-invasive (A) and anti-migration (B) effects of IC_{50} concentrations of bortezomib (IC_{50}: 101.65 nM) and ixazomib (IC_{50}: 77.802 nM) on HUVEC (mean ± SD, n = 6, $p < 0.05$*, $p < 0.0001$****).

the control group in the EGFR and COX10 genes, both of which have been implicated in the process of metastasis ($p < 0.0001$****). The study revealed that the mRNA expression levels of NF-kB1, NF-kBIA, TP-53, and COX10 were found to be reduced in both the bortezomib and ixazomib groups when compared to the A549 + M2c macrophage co-culture group. In the M2c + A549 Bortezomib experimental group, it was observed that the expression levels of NF-kB1 ($p < 0.05$*) and NF-kBIB ($p < 0.001$***) genes were comparatively reduced compared to the control group.

Based on our findings, it has been determined that the mRNA expression levels of VEGFA ($p < 0.001$***), VEGFB ($p < 0.01$**), EGFR ($p < 0.0001$****), MMP9 ($p < 0.0001$****), ANGPT1 ($p < 0.001$***), ANGPT2 ($p < 0.0001$****), and IL-6 ($p < 0.01$**) genes, which are closely associated with the process of angiogenesis, exhibited an increase in the M2c macrophage co-culture group when compared to the HUVEC control group. In the M2c + HUVEC Bortezomib experimental group, it was observed that the expression levels of MMP9 and IL-6 genes were comparatively reduced compared to the control group ($p > 0.05$ n.s). One of the significant findings in our data is the observed decrease in VEGFB mRNA expression levels in both the bortezomib ($p < 0.01$**) and ixazomib ($p < 0.05$*) treatment groups when compared to the control group.

Previous studies have shown that co-culturing macrophages with lung cancer cells can alter gene expression profiles [3, 35, 44, 45]. The NF-kB family consists of five subunits (RelA, c-Rel, RelB, NF-

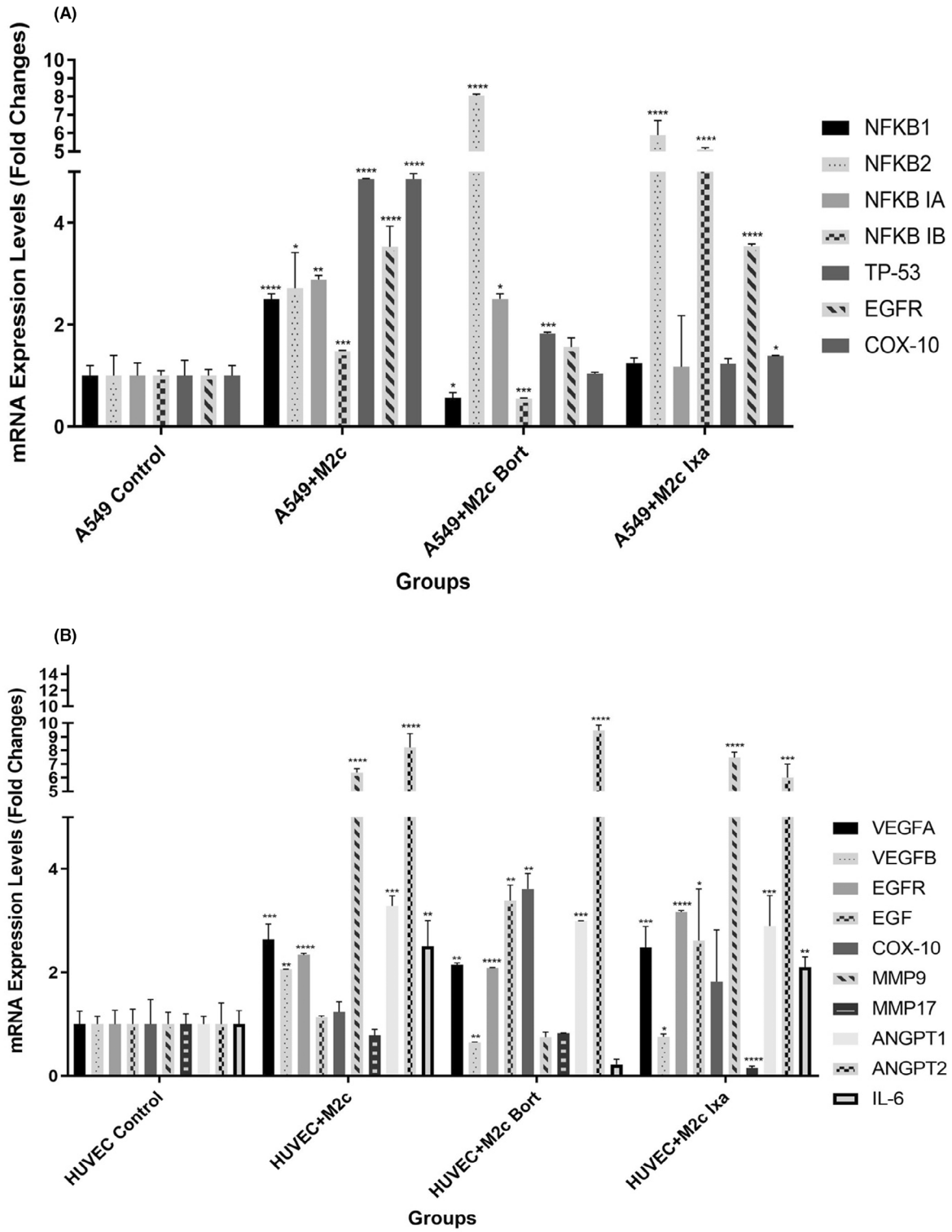

Fig. 7. RT-PCR analysis for gene expression levels of A549 (A) and HUVEC (B) cells. The data represent the means ± SDs of three individual experiments (n = 3 for each). A one-way ANOVA was used for statistical analysis for each group, followed by a post hoc Tukey's multiple comparisons test. All of the results are displayed on a single graph. No difference: $p > 0.05$; significant difference: $p < 0.05$*, $p < 0.01$**, $p < 0.0001$**** vs. untreated controls.

kB1, and NF-kB2) and is involved in numerous biological processes such as inflammation, immunity, cellular differentiation, growth, tumorigenesis, and programmed cell death by controlling the expression of many genes [46]. TP53 is a multifaceted protein that performs various functions in the development of tumors, including the regulation of cell cycle progression, invasion, migration, epithelial-mesenchymal transition (EMT), and other related processes [47]. Macrophages play a pivotal role in the pathogenesis of inflammatory disorders. Furthermore, they promote angiogenesis by secreting pro-angiogenic cytokines and growth factors such as VEGF [42]. VEGF-A, a critical regulator of angiogenesis, is consistently expressed by macrophages and exhibits a significant upregulation following macrophage activation [48]. VEGF is a molecule that has been extensively investigated in the context of tumor angiogenesis [49]. Nevertheless, additional research is needed to explore other molecules that are associated with this process. In humans, angiopoietin-1 is a member of the ANGPT-TIE protein family, which includes three ligands (ANGPT1, ANGPT2, and ANGPT4) and two receptors (TIE1 and TIE2). ANGPT1 is widely recognized as the primary mediator of the pro-angiogenic signaling pathway, which operates independently of VEGF. It has been reported that extensive research on their role in the tumor angiogenesis process is still ongoing [50]. Macrophages are capable of initiating matrix remodeling processes that facilitate the migration of endothelial cells and contribute to the overall remodeling of blood vessels. In particular, MMP9 becomes important in this process [51]. The identification of multiple genes involved in various angiogenesis processes that facilitate the elevation of mRNA levels in M2c macrophages after polarization with hydrocortisone is a notable conclusion of our research. It is also noteworthy that VEGFB, an essential marker of angiogenesis, was successfully inhibited by the proteasome inhibitors that we used in the study. For ongoing investigations, this conclusion has important implications.

CONCLUSIONS

The process of metastasis and angiogenesis in cancer is influenced by various factors, including the genetic and molecular features of cancer cells as well as their interaction with the immune system and the tumor microenvironment. Prior research predominantly concentrated on M1 macrophages, while investigations pertaining to M2 macrophages, particularly the M2c subtype, were found to be limited in terms of comprehensive studies. Our investigation revealed that the co-culture of M2c macrophages and A549/HUVEC resulted in an increase in invasion and angiogenesis. This effect was particularly linked to the increase in gene expression in the NFKB and VEGF families. Moreover, we hypothesize that MMP9 and ANGPT may serve as potentially relevant mechanisms for M2c-induced angiogenesis. However, additional investigation is necessary to fully elucidate the precise mechanism underlying M2-induced angiogenesis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

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