Proteasome Inhibitor Immunotherapy for the Epithelial to Mesenchymal Transition: Assessing the A549 Lung Cancer Cell Microenvironment and the Role of M1, M2a and M2c 'Hydrocortisone-Polarised' Macrophages

Selin Engür Öztürk (✉ selinengur@gmail.com)

Pamukkale University <https://orcid.org/0000-0003-1534-8117>

Miriş DİKMEN

Anadolu Üniversitesi: Anadolu Üniversitesi

Research Article

Keywords: A549, Macrophages, EMT, NF-κB, Bortezomib, Ixazomib

Posted Date: January 3rd, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1068010/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Lung cancer is a leading cause of cancer-related deaths, primarily as a result of metastases. In this metastasis, the epithelial-to-mesenchymal transition (EMT) is essential. Interaction with the cancer cell microenvironment is primarily dependent on M1- and M2-polarized macrophage. In this study, we revealed the EMT-associated activity of M1, M2a and M2c macrophages in A549 lung cancer cells. We established a co-culture model of A549 lung cancer cells utilizing THP-1-derived M1/M2 polarised macrophages to explore the involvement of macrophages in the immune response, apoptosis, and EMT in lung cancer. Although multiple polarising agents are routinely used for M1 and M2 conversion, we assessed a new possible polarising agent, hydrocortisone. M1 increased A549 cell sensitivity to proteasome inhibitors and decreased A549 cell viability by inducing apoptosis. EMT was induced in the presence of M2c macrophages in A549 cells by the levels of vimentin, fibronectin, E-cadherin, NF- κ B, CCL-17. We also revealed the antiproliferative effects of bortezomib and ixazomib on A549 cells in both 2D and 3D cultures. Our findings could help develop an immunotherapeutic strategy by shedding light on a previously undiscovered part of the EMT pathway. Furthermore, additional investigation may reveal that polarising tumour-associated macrophages to M1 and eliminating the M2a or particularly the M2c subtype are effective anti-cancer strategies.

Introduction

Cancer is defined as uncontrolled cell proliferation caused by genetic and epigenetic disorders, and it is the world's second most common cause of death after cardiovascular system disorder. The 'hallmarks of cancer' include cell death resistance, the ability to evade immunological surveillance, triggered angiogenesis, invasion and metastasis activation, and others [1, 2]. The primary goal of cancer research today is to discover different early detection technologies, as well as to investigate the molecular pathways of lung tumour progression, particularly specific mechanisms of metastasis [3].

Non-cancer cells and tumor tissue structures are defined by the microenvironment, and tumor-associated macrophages make up 5–40% of the tumor mass in solid tumors. [4, 5]. Tumour-associated macrophages are central in the relationship between chronic inflammation conditions in cancer [6, 7] and play an vital role in the progression of tumors, epithelial-mesenchymal transition (EMT), angiogenesis, cancer metastases, and drug resistance [4].

EMT is a morphological transformation of cells from an epithelial-polarized phenotype to a mesenchymal fibroblastoid phenotype that plays a critical role in cancer metastasis [8]. In the development process, a balanced epithelial-mesenchymal transition is essential, while abnormal EMT contributes to pathogenesis by disrupting epithelial homeostasis for diseases such as cancer [9]. The nuclear factor κ B (NF- κ B) is a significant marker of cancer invasion and EMT [10, 11]. NF- κ B is a transcription factor that regulates gene-encoding molecules such as chemokines, apoptosis, surface receptors in the immune response, cytokines, cell adhesion molecules, and inflammation [12]. Several therapeutic approaches have been proposed in recent years, focusing on the NF- κ B axis targeting tumour invasion.

We utilised 2D and 3D cell culture approaches to evaluate the effects of bortezomib and ixazomib, recently developed therapeutic strategies, on antiproliferative, apoptotic and EMT pathways in A549 lung cancer co-cultures that included THP-1-derived M1, M2a and M2c polarised macrophages. This is the first article of M2c macrophage polarization by hydrocortisone that we are aware of.

Materials And Methods

Chemicals

Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), Eagle's Minimum Essential Medium (EMEM), Fetal bovine serum (FBS), hydrocortisone, Hoechst 33258 dye, RPMI-1640 medium, phosphate-buffered saline (PBS), phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), interferon- γ (IFN- γ), interleukin-4 (IL-4) and interleukin-13 (IL-13) were purchased from Sigma Aldrich (St. Louis, MO, USA). E-cadherin monoclonal antibody (E-cadherin [4A2] mouse mAb) and secondary antibody anti-mouse Alexa Fluor 488 (ab150113, Abcam), vimentin monoclonal antibody (vimentin [D21H3] XP® Rabbit mAb) and secondary anti-rabbit Alexa Fluor 594 (anti-rabbit IgG [H+L], F(ab')₂ fragment [Alexa Fluor® 594 Conjugate]) were purchased from Cell Signaling (Danvers, MA, USA).

Cell culture and treatments

A549 non-small cell lung cancer cells (CCL-185, ATCC®, USA), CCD-19Lu human lung fibroblast cells (CCL-210, ATCC®, USA) and THP-1 human monocyte cells (TIB-202, ATCC®, USA) were cultured in RPMI-1640 medium with 1% penicillin-streptomycin and 10% FBS (Sigma; St. Louis, MO, USA). Cultures were incubated at 37°C in a humidified CO₂ (5%) incubator (Heracell™, Thermo Fisher Scientific™, Pittsburgh, PA, USA). Bortezomib (BioChemica, Sauerlach, Germany) and ixazomib (Active Biochem, Germany) were dissolved in DMSO and diluted with medium to the required concentrations. The control group was incubated in 0.1 percent DMSO-containing medium.

Polarisation of macrophages into M1, M2a and M2c subtypes

THP-1 cells were treated with 100 ng/mL PMA in serum-free medium (SFM) for 24 hours to induce differentiation into a macrophage-like phenotype. To obtain the macrophage (M0) resting state, differentiated adherent cells were rested in the culture medium (without the addition of PMA, but with 10% FBS and 1% penicillin-streptomycin). for another 48 hours. It was discovered that macrophages polarized into M1, M2a, or M2c after being exposed to 20 ng/mL LPS plus IFN- γ for 24 hours, 25 ng/mL IL-4 plus IL-13 for 72 hours, and 1 M hydrocortisone for 72 hours. To remove carry-over cytokines, LPS, and hydrocortisone, polarised macrophages were thoroughly washed and incubated in fresh media for 24 hours. The expression of cell surface markers CD80, CD-206, CD23 and CD163 (BioLegend, San Diego, CA, USA) were used to decide the macrophage subspecies using a BD Accuri C6 flow cytometry. The mRNA expression levels of some genes (IL-1 β , IL-8, IL-18, CXCL-1, CXCL-3, CCL-22, CCL-24, TGF- β) were determined using a real-time polymerase chain reaction (RT-PCR) method to show the polarisation of M0,

M1, M2a and M2c subtypes. Finally, macrophages M0, M1, M2a and M2c were photographed with the Leica DM inverted light microscope to assess morphology.

WST-1 cytotoxicity test

WST-1 assay (Roche) was used to determine cell viability. M0 macrophages were cultured at a density of 1×10^4 cells per well into 96-well plates. Cells were treated with bortezomib and ixazomib at different concentrations for 24 and 48 hours. After incubation, wells were filled with WST-1 cell proliferation reagent (10 μ l/well) and absorbances (420 nm) were measured after 3 hours. The absorbance of the samples was measured using a Cytation 3 Multi-mode reader. (BioTek, Winooski, VT, USA). The number of living cells was directly proportional to the absorbance, and cell viability was expressed as a percentage of controls [13, 14].

Proliferation assay by real-time cell analysis system (RTCA DP)

The RTCA DP system monitors cell behavior in real-time by measuring changes in impedance of attached cells and providing a cell index (CI) value. During the experiment days, the impedance of the wells for each group was measured every hour and the impedance of the wells for each concentration was measured every hour during the experiment days and defined as a CI value. We searched at cell proliferation as well as the IC_{50} concentrations of bortezomib and ixazomib. First, the background of the 16-well E-plates (Roche Applied Sciences, Indianapolis, IN, USA) was measured in 100 μ l RPMI 1640 medium by the RTCA DP. The cells were then seeded at a density of 1×10^4 per well in 16-well E-plates in 100 μ l medium. After 24 hours of incubation, the impedance of each well was measured at 1-hour intervals using the xCELLigence RTCA DP Instrument (ACEA Biosciences, San Diego, CA, USA). Lastly, the instrument was stopped, and 100 μ l of medium was removed from the wells containing bortezomib and ixazomib at several concentrations. All assays were performed in 8 wells, and the average values were calculated. Cell proliferation and IC_{50} concentrations of bortezomib and ixazomib were monitored and evaluated for 48 h according to the CI values by RTCA DP Software 1.2.1 [15, 16]

Co-culture procedures

THP-1 polarised macrophages were co-cultured with A549 lung cancer cells in indirect contact via transwell inserts (Corning, NY, USA) to investigate the impact of M1, M2a and M2c on cancer cell sensitivity to proteasome inhibitors. The A549 cells were co-cultured with M1, M2a, and M2c macrophages in six-well plate cell culture inserts with a 0.4- μ m 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 A549 cells plated in the lower chamber. The THP-1 cells were seeded into the transwell apparatus's upper chamber, stimulated to differentiate into M0 macrophages by adding 100 ng/mL PMA for 24 h, washed with PBS, and incubated for another 48 h to eliminate the effect of PMA. The attached cells, which corresponded to M0 macrophages, were polarised into M1, M2a, or M2c macrophages by

adding 20 ng/mL LPS plus IFN- γ for 24 h, 25 ng/mL IL-4 plus IL-13 for 72 h, and 1 μ M hydrocortisone for 72 h. THP-1 monocyte differentiation for M1, M2a, and M2c macrophages was started on different days to obtain differentiated and polarised macrophages on the same day. A549 cells were seeded in the lower chamber 24 hours before macrophage polarisation ended. The upper chambers containing the M1, M2a, or M2c macrophages were then placed directly on top of the A549 cells in the six-well plates. The two cell populations were incubated for 48 hours with bortezomib and ixazomib IC₅₀ concentrations added directly into the wells.

Real-time monitoring of A549 cell proliferation under co-culture conditions with M1, M2a or M2c polarised macrophages using the E-plate insert

The E-Plate insert allows for real-time analysis of specific cell-cell interactions while keeping the cells separated in compartments. A 0.4 μ m pore size membrane separates two different cell populations, allowing control of physical contact and interaction duration. The xCELLigence instrument was used to continuously monitor cell proliferation (Roche, Mannheim, Germany). Briefly, A549 cells were seeded in E-plate 16 (ACEA Biosciences, San Diego, CA) at a density of 1×10^4 /well with culture medium and incubated in the RTCA cradle for 24 hours. Until the end of the experiment, hourly impedance signals were recorded. After 24 hours of incubation, the E-plates were taken to the RTCA unit where 160 μ L of culture medium was replaced with 80 μ L of SFM for the control. (For the bortezomib and ixazomib groups, IC₅₀ concentrations are determined in SFM). The inserts were gently lowered into the E-16 plate (the insert consists of 80 μ L SFM with M1, M2a or M2c polarised macrophages). The control group consisted of a well with no cells seeded in the same insert(s). The impedance recording was then resumed on the device for the duration of 48 h. Four wells were used for each treatment group.

Apoptosis detection using Annexin V-FITC and propidium iodide (PI) staining

A549 cells (2×10^5 /well) were seeded in 6-well plates 24 hours before the end of macrophage polarisation process. A549 cells and macrophages were co-cultured in SFM at a cell density of 1:5. A549 cells and M1, M2a or M2c co-culture groups were incubated with bortezomib and ixazomib IC₅₀ concentrations for 48 hours. A549 cell analysis was conducted according to the Annexin V-FITC Apoptosis Detection Kit procedure, used to detect apoptosis as described previously [13]. Finally, the samples were diluted with 250 μ L of the binding buffer, processed for data acquisition, and analysed on a BD Accuri™ C6 Flow Cytometry; at least 1×10^4 cells were analysed per sample.

Detection of caspase-3 activity

Caspase-3 is an essential protease that initiates apoptosis [17]. Alterations of the caspase-3 level of cells were determined using the PE Active Caspase-3 Apoptosis Kit (BD Pharmingen, Catalogue Number: 550914). A549 cells (2×10^5) were seeded in 6-well plates 24 hours before the end of polarisation process. A549 cells and M1, M2a or M2c co-culture groups were incubated with bortezomib

and ixazomib IC₅₀ concentrations for 48 hours. After incubation, the samples were analyzed using BD Accuri™ C6 Flow Cytometry. At least 1 x 10⁴ cells were analysed per sample.

Determination of loss of mitochondrial membrane potential by JC-1 dye using flow cytometry

We examined the loss of mitochondrial membrane potential in response to ixazomib and bortezomib in cells by the Flow Cytometry Mitochondrial Membrane Potential Detection Kit (BD Pharmingen, Catalogue Number: 551302). A549 cells (2x10⁵) were seeded in 6-well plates 24 h before the end of polarisation. A549 cells and M1, M2a or M2c co-culture groups were incubated with bortezomib and ixazomib IC₅₀ concentrations for 48 h. After incubation, the samples were analyzed using BD Accuri™ C6 Flow Cytometry. At least 1 x 10⁴ cells were analysed per sample [13, 17].

Cytokine detection

A549 cells (2 x10⁵) were seeded in 6-well plates 24 hours before the end of polarisation. A549 cells and M1, M2a or M2c co-culture groups were incubated with bortezomib and ixazomib IC₅₀ concentrations for 48 h. At the end of the incubation, supernatants were collected from co-culture. The BD human inflammatory kit procedure was used to measure TNF- and IL-10 cytokine levels (BD™ Cytometric Bead Array [CBA] Human Inflammatory Cytokines Kit, Catalogue Number: 551811). An FCAP array was used in a BD Accuri C6 flow cytometer for CBA analysis.

Quantitative RT-PCR

Total RNA was extracted from M1, M2a, or M2c macrophages to determine the expression of M1 and M2 macrophage markers, from A549 lung cancer cells to assess single-cell control, and from co-cultured A549 cells and macrophages to determine the change in genes in the EMT pathway. RNA was extracted using a MagNA Pure Compact RNA Isolation Kit for the MagNA Pure Compact Instrument LC 2.0 system (Roche Diagnostics, Mannheim, Germany). Reverse transcription was performed using a High-Fidelity cDNA Synthesis Kit (Roche, Germany). A NanoDrop spectrophotometer was used to verify the RNA samples' purity. For cDNA synthesis, 500 ng of RNA were taken from each RNA population. Real-time PCR was performed using the SYBR Green Master Mix (Roche Applied Science, Germany) on a LightCycler® 480 instrument (Roche Applied Science, Germany). The internal control was glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Table 1 shows the primer sequences that were used. The results were normalized to GAPDH expression levels and presented as averages of three experiments.

Table 1
Real-Time nucleotide primer sequence. Transcripts Primer forward Primer reverse

Genes	Short name	Forward Primer- Reverse Primer
Interleukin-1 Beta	IL-1 β	Forward: 5'-AAGATGCTGGTTCCTGCC-3' Reverse: 5'-GCGTGCAGTTCAGTGATCGTAC-3'
Interleukin -8	IL-8	Forward: 5'-CCGGAAGGAACCATCTCACT-3' Reverse: 5'-ACTTCTCCACAACCCTCTGC-3'
Interleukin -18	IL-18	Forward: 5'- GATAGCCAGCCTAGAGGTATGG-3' Reverse: 5'-CCTTGATGTTATCAGGAGGATTCA -3'
Chemokine Ligand-1	CXCL-1	Forward: 5'- AGCTTGCCTCAATCCTGCATCC-3' Reverse: 5'- TCCTTCAGGAACAGCCACCAGT-3'
Chemokine Ligand-3	CXCL-3	Forward: 5'-TTCACCTCAAGAACATCCAAAGTG-3' Reverse: 5'-TTCTTCCCATTCTTGAGTGTGGC -3'
Transforming Growth Factor Beta	TGF-Beta	Forward: 5'- TACCTGAACCCGTGTTGCTCTC-3' Reverse: 5'- GTTGCTGAGGTATCGCCAGGAA-3'
Chemokine Ligand-22	CCL-22	Forward: 5'- TCCTGGGTTCAAGCGATTCTCC-3' Reverse: 5'- GTCAGGAGTTCAAGACCAGCCT-3'
Chemokine Ligand-24	CCL-24	Forward: 5'- TGAGAACCGAGTGGTCAGCTAC-3' Reverse: 5'- TTCTGCTTGGCGTCCAGGTTCT-3'
Glyceraldehyde-3 phosphate dehydrogenase	GAPDH	Forward: 5'- GTCAAGGCTGAGAACGGGAA -3' Reverse: 5'- AAATGAGCCCCAGCCTTCTC -3'
Vimentin	VIM	Forward: 5'- GGACCAGCTAACCAACGACA -3' Reverse: 5'- AAGGTCAAGACGTGCCAGAG -3'

Genes	Short name	Forward Primer- Reverse Primer
Fibronectin	FN1	Forward: 5'- CCCTGATTGGAACCCAGTCC -3' Reverse: 5'- CTGTCACACGAGCCCTTCTT -3'
E-Cadherin	CDH1	Forward: 5'- AGCTCTGCTGCCTCGTCTAT -3' Reverse: 5'- GGCCTCTCTTGGTTAGGAGG -3'
Nuclear Factor Kappa B	NF-kB	Forward: 5'- CGCCGCTTAGGAGGGAGA -3' Reverse: 5'- GGTATGGGCCATCTGCTGTT -3'
Vascular Endothelial Growth Factor	VEGF-A	Forward: 5'- GCAAGACAAGAAAAATGTGACAA-3' Reverse: 5'- TGGTTTCTGTATCGATCGTTCT- 3'
Chemokine ligand -17	CCL-17	Forward: 5'- TGTTCGGACCCCAACAACAA -3' Reverse: 5'- TAGTCCCGGGAGACAGTCAG -3'

Immunofluorescence staining of vimentin and E-cadherin

Vimentin and E-cadherin levels in the A549 control and M2a and M2c co-culture groups were analysed by flow cytometry (Accuri C6, BD). The M2c macrophage co-culture groups found to be more significant, vimentin and E-cadherin levels were investigated by immunofluorescence staining in the Cytation 3 Cell Imaging Multimode Reader. An Immunofluorescence Application Solutions Kit (Cell Signaling) was used for the fixation, permeabilisation and immunostaining of antibodies to determine the levels of E-cadherin and vimentin in the cells. The vimentin antibodies (vimentin [D21H3] XP® rabbit mAb, Cell Signaling) were diluted 1:100 and E-cadherin monoclonal antibody (E-cadherin [4A2] mouse mAb, Cell Signaling) was diluted 1:50 in the same solution. The cells were incubated with 100 µl of antibody overnight at 4°C. After incubation, cells were rinsed twice with PBS and secondary antibody Anti-rabbit Alexa Fluor 594 (anti-rabbit IgG [H+L], F(ab')₂ Fragment, Cell Signaling) at a 1:500 dilution. Secondary antibodies (anti-mouse Alexa Fluor 488, ab150113, Abcam) were added to the wells and incubated for 1 hour at room temperature. Hoechst 33258 (10 µg/ml) was used to stain the cell nuclei (blue) 10 minutes before imaging with Cytation 3 Cell Imaging Multi-Mode Reader (Bio-Tek, USA) [18].

3D culture studies

Culturing cells with AlgiMatrix

In 96-well 3D alginate scaffold plates, cells (2×10^4 /well) were incorporated into a 3D alginate scaffold in 100 mL of media to determine the optimal time for the formation of spheroids. After 20 minutes, a further 100 mL of media was added, and the cells were grown in an incubator at 37°C with 5% CO₂ for another 20 minutes. Every 48 hours, the media was changed. The formation of tumor spheroids in 3D alginate

scaffold wells was observed at 3, 5, and 7 days after cell seeding to assess growth. Images were taken using an inverted microscope (Leica DM 300), and the size of spheroids was determined by the Leica LAS Analysis program. Also, cell spheroids were imaged by Hoechst fluorescent dye using BioTek Cytation 3 Imaging Multi-mode Reader.

Comparison of 2D and 3D cytotoxicity data of bortezomib and ixazomib

Based on the preliminary experiments, the more abundant spheroid formation was observed in the 7-day culture. As a result, 7 day was chosen as the starting point for treatment. Bortezomib and ixazomib at concentrations ranges of 0.1–400 μM were used for the treatment. After 48 h incubation, for WST-1 viability determination, sponges were dissolved using an AlgiMatrix Dissolving Buffer, which provides a quick and straightforward way to recover cells from the AlgiMatrix bioscaffold. After 3 hours, wells were filled with the cell proliferation reagent WST-1 and absorbances were measured. 2D culture studies were also conducted in parallel with this experiment. IC_{50} values were then calculated for both 2D and 3D cultures using MS Excel.

Statistical Analysis

The results were analyzed using one-way ANOVA and Tukey's post hoc test in Graphpad Prism 6.0. The means of three independent experiments are expressed as mean standard deviation ($\pm$). The significance of the results in comparison to the control group is represented by P values ($P > 0.05$ n.s, $P < 0.05^*$, $P < 0.01^{**}$, $P < 0.001^{***}$, and $P < 0.0001^{****}$).

Results

Monocytic THP-1 cells differentiation into M0 macrophages

THP-1 cells grew in suspension as single cells or partially in clusters and had a wide and round morphology (**Figure A1**). THP-1 cells were differentiated into macrophages after 24 hours of stimulation with 100 ng/mL PMA. During differentiation, the cells attached to the flask bottom changed shape from round to almost spindle-shaped, and pseudopods appeared in some cells. Due to the fact that all macrophage populations express CD68, different PMA concentrations and incubation times were used to determine differentiation. Based on our flow cytometry results, we chose a 24-hour incubation with 100 ng/mL PMA, followed by a 48-hour rest period (CD-14 [5.5%] and the CD-68 [58.2%] levels were determined, data not shown).

THP-1 polarisation into pro-inflammatory M1 macrophages

In the involvement of IFN- γ and in combination with inflammatory stimuli such as LPS, macrophages polarize to the M1 phenotype, according to numerous studies [19–23]. Based on these data, we tested various concentrations of LPS (20 ng/mL or 1 $\mu\text{g/mL}$), combined with 20 ng/mL of IFN- γ , and incubated THP-1 macrophages for 24 h. Macrophage M1 polarisation was assessed by measuring the mRNA

expression of M1 markers, pro-inflammatory cytokines IL-1 β , IL-8, IL-18, CXCL1 and CXCL3, using RT-qPCR and CD80 membrane receptors level by flow cytometry. Based on our CD80 results, 20 ng/mL IFN- γ + 20 ng/mL LPS was selected for M1 polarisation to be applied during the 24-hour incubation time (**Figure B1**). An increased pro-inflammatory marker (IL-1 β , IL-8, IL-18, CXCL1 and CXCL3) expression profile was obtained by incubation with IFN- γ combined with 20 ng/mL LPS in comparison to M0 macrophage (**Figure C1**). Additionally, we examined the mRNA expression of some M2 markers (TGF-Beta, CCL22, and CCL24) in M1 macrophages and found that these genes were not significantly expressed. Finally, incubating THP-1 macrophages (M0) for 24 hours with IFN- 20 ng/ml and LPS 20 ng/mL causes them to polarize into M1 macrophages.

THP-1 polarisation into anti-inflammatory M2a and M2c macrophages

In several studies, M2a polarisation has been reported to occur in the presence of cytokines IL-4 and IL-13 [24–26]. The concentrations of IL-14 and IL-13 used in these studies were in the range of 20-25 ng/mL, and incubation times were 24, 48 and 72 hours [22, 27]. In the limited amount of previously published data, especially on M2c polarised macrophages, IL-10 (20 ng /mL) has been used during the differentiation of THP-1 cells into M2c macrophages [21, 23]. No previous study has utilised hydrocortisone to polarise M2c cells. We incubated M0 macrophages with IL-4 and IL-13 at a concentration of 25 ng/ml or hydrocortisone at 1 and 10 μ M for 24 or 72 h. The M2a and M2c phenotype was characterised by studying CD206 (M2 marker), CD23 (M2a marker) and CD206, CD163 (M2c marker) membrane receptor levels by flow cytometry, respectively (**Figure A1**). Incubation of THP-1 macrophages (M0) with IL4 and IL-13 (25 ng/ml) and hydrocortisone (1 μ M) for 72 h induces their polarisation into M2a and M2c macrophages, respectively. Furthermore, mRNA expression levels of some M2 markers (TGF-Beta, CCL22 and CCL-24) were increased in M2a and M2c macrophages compared to M0 macrophage (**Figure C1**).

Antiproliferative effects of bortezomib and ixazomib determined by RTCA DP

RTCA DP detects the cell number indirectly by measuring electrical impedance and generates real-time data by collecting measurements at predetermined intervals over a period of days [14–16]. In our study, the RTCA DP system was used for the real-time and time-dependent analysis of IC₅₀ concentrations of A549 and CCD-19Lu cells. The WST-1 method was used to select the appropriate concentrations of bortezomib and ixazomib for evaluation in the xCELLigence system (data not shown). **Figures A2, B2, C2 and D2** show that bortezomib and ixazomib decreased the viability of A549 and CCD-19Lu cells in a dose-dependent manner. Also, to compare the cytotoxic effects of the bortezomib and ixazomib on A549 lung cancer and CCD-19Lu healthy lung fibroblast cells, their IC₅₀ values were calculated after 48 h exposure. The IC₅₀ value determined for bortezomib and ixazomib in A549 lung cancer cells was found to be lower than the IC₅₀ value determined for CCD-19Lu cells. IC₅₀ concentrations of bortezomib and ixazomib (45 nM and 2 μ M, respectively) were determined and applied to macrophages during the co-

culture process, and macrophage viability was determined by the WST-1 method. Bortezomib and ixazomib had no significant cytotoxic effect on M0 macrophages at 24 and 48 h incubation (**Figure E2**).

M1 macrophages reduced A549 cell viability and proliferation while increasing drug sensitivity to bortezomib and ixazomib.

Numerous studies demonstrate that interactions with tumor-associated macrophages can influence cancer cell proliferation and chemosensitivity [23]. There is no other study using macrophage polarization co-culture and a real-time cell analysis system in the literature. The E-plate insert allows for real-time cell-cell interactions while keeping cells apart. Proliferation results were determined more precisely because A549 cells were located in the same culture medium as the E-plate insert used in co-cultures with M1, M2a or M2c polarised macrophages and their proliferation was measured in real-time (**Figure A3, B3, C3, D3**). A549 and M1 macrophage co-culture groups significantly decreased A549 cell viability and proliferation relative to the A549 control (**Figure A3**). Also, the results showed that the antiproliferative effects of bortezomib and ixazomib were higher in the A549 and M1 macrophage co-culture groups (**Figure B3**). We used annexin V/PI staining, caspase-3 activation, and mitochondrial membrane potential assays to determine whether the reduced cell number of A549 cells exposed to M1 was due to apoptosis. M1 co-culturing for 48 hours induced apoptosis in A549 cells, according to annexin V/PI staining. When A549 cells were incubated with M1 macrophages in the lack of drugs, there was an increase in apoptosis. Notably, bortezomib and ixazomib induced apoptosis most frequently in the M1 macrophage co-culture group (**Figure E3**). When A549 cells were incubated with M1 macrophages, the level of caspase-3 was higher than when the cells were incubated alone. M1 macrophages also significantly increased caspase-3 activity relative to the control group incubated with bortezomib and ixazomib without macrophages. M1 macrophages also increased caspase-3 activity in A549 cells treated with bortezomib and ixazomib (**Figure F3**). The observed loss of mitochondrial depolarisation also supports these findings (**Figure G3**). When A549 cells were incubated with M2a or M2c macrophages, the apoptosis of cancer cells did not change compared to the control group. Furthermore, JC-1 depolarisation was much less common in cells incubated with M2, particularly M2a. However, M2a and M2c macrophages increased the A549 cell apoptosis induced by bortezomib and ixazomib for all three apoptosis-related experiment results (**Figure E3, F3 and G3**). Overall, these findings show that M1, M2a, and M2c macrophages in A549 lung cancer cells modulated the apoptotic response to proteasome inhibitors bortezomib and ixazomib. M1 macrophages alone inhibited cell proliferation and increased drug-induced apoptosis. In the absence of drugs, however, M2 macrophages were ineffective or reduced apoptosis in A549 cells.

M2c macrophages induced the EMT of lung A549 cancer cells

To determine whether the EMT pathway was activated, TNF- α and IL-10 cytokine levels were determined from A549 cell supernatants and M1, M2a and M2c polarised macrophage co-culture groups using flow cytometry. IL-10 cytokines increased in co-culture groups formed by both M2a and M2c polarised macrophages and A549 cells, and there was a significant increase in the M2c macrophage co-culture

group ($p<0.01^{**}$) (**Figure A4**). mRNA expression levels of EMT-related factors (vimentin, fibronectin, E-cadherin, NF- κ B, VEGF and CCL-17) in A549 cells were evaluated in a co-culture model of M1, M2a and M2c polarised macrophages. The most important mesenchymal factors, vimentin and fibronectin, increased, and E-cadherin decreased in both A549 M2a and M2c macrophage co-culture groups. Also, no significant changes in vimentin, fibronectin, E-cadherin, NF- κ B, VEGF and CCL-17 mRNA expression were found in the M1 macrophage groups. mRNA expression levels of CCL-17 did not change significantly in the M1 polarised macrophage co-culture group, but they decreased in the M2a macrophage group compared to the A549 control group ($p<0.05^{*}$). A significant increase ($p<0.0001^{****}$) in the M2c macrophage co-culture group was distinct as it indicated that EMT increased the most in the M2c polarised macrophage co-culture group (**Figure B4**). Based on these findings, it was determined that EMT was induced, in particular, in M2c macrophage A549 co-culture groups. This result is supported by the increased vimentin and decreased E-cadherin levels determined by flow cytometry (**Figure C4**). Proteasome inhibitors have also been found to reduce EMT by decreasing vimentin, NF κ B, VEGF and CCL-17 expression levels. Especially for ixazomib, the reduction of EMT was statistically more significant than bortezomib (**Figure D4**). In addition, vimentin and E-cadherin in M2c co-culture groups, as imaged by immunofluorescence staining images (**Figure E4**), show an increase in vimentin (red) and a decrease in E-cadherin (green) monoclonal antibody levels in the A549 and M2c co-culture group compared to the A549 control group. Finally, it was determined that bortezomib and, more so, ixazomib decreased M2c-induced EMT in A549 lung cancer cells.

Optimisation of In Vitro 3D A549 Lung Cancer Tumor Model

A 3D culture environment was created using the AlgiMatrix® 3D Culture System. Based on the type of cells incorporated, the spongy features of the AlgiMatrix® system provides the needed place for cells to grow as models of *in vitro* solid tumors include lung cancer [28]. **Figure A5** shows spheroid areas and morphological images of randomly selected A549 cells on days 3, 5 and 7. The graph generated by the average spheroid area (μm^2) was obtained from A549 cell images taken on different days (**Figure B5**). The spheroid area increased statistically on day 7 compared to days 3 and 5 ($p < 0.0001^{****}$).

Comparative analysis of IC₅₀ values of bortezomib and ixazomib in 2D and 3D systems

3D cultures are known for accurately replicating the physiologic microenvironment and having a high level of harmony with *in vivo* conditions. In our study, we aimed to determine IC₅₀ concentrations for proteasome inhibitors to shed light on *in vivo* models by taking advantage of 3D culture. The IC₅₀ values for bortezomib and ixazomib were significantly higher in AlgiMatrix systems than in 2D culture models. After 48 hours of A549 incubation with bortezomib and ixazomib in 2D and 3D culture, the IC₅₀ value in 3D culture was approximately 15 times higher for bortezomib and 9 times higher for ixazomib than in 2D culture (Table 2).

Table 2. IC₅₀ values of bortezomib and ixazomib in A549 cells at 48 h in 2D and 3D cell culture.

IC ₅₀ concentration (μM)	2D	3D
Bortezomib	6.32 μM	98.6 μM
Ixazomib	41,27 μM	353.01 μM

Discussion

The initiation and metastasis of lung cancers depend not only on the genetic and molecular characteristics of the cancer cells but also on their interaction with the immune system and the tumour microenvironment [29]. In solid tumours, TAMs comprise 5–40 percent of the total tumor mass in most cases. Furthermore, there is usually a link between the number of TAMs and the specific condition, depending on the type of tumor [5]. In response to a stimulus in their environment, macrophages can be differentiated into M1 or M2 macrophages. Recent research suggests that macrophage polarization may be used as a therapeutic strategy in the treatment of cancer and angiogenesis. [4, 23]. Previous studies have mostly focused on M1 macrophages, and no subtype distinction has been observed in M2 macrophages. There are limited data available on M2c macrophages. In this study, we found that macrophages with M1, M2a, and especially M2c polarization had different effects on lung cancer cell apoptosis, the EMT pathway, and gene expression profiles.

Notably, we used hydrocortisone for the first time in M2c polarisation by focusing more on M2 macrophages. M2 macrophages are polarised to M2c macrophages by different stimuli. Although M2c macrophages are reported to be polarised with IL-10, TGF- and glucocorticoids [30, 31], no studies to date have reported polarisation using the glucocorticoid hydrocortisone. We optimised hydrocortisone polarisation of M2c for the first time in this study, identifying many surface markers (CD206 and CD163) and cytokines and observing chemokine gene expression (IL-1 β , IL-8, IL-18, CXCL-1, CXCL-3, CCL-22, CCL-24, TGF- β).

Studies have shown that M1 macrophages promote to the inhibition of tumour growth and increase the sensitivity of chemotherapy agents [23, 27]. We found that M1 polarised macrophages alone decreased A549 cell viability using the real-time cell analysis system. In addition, M1 macrophages alone affected and increased the bortezomib and ixazomib-induced apoptosis. Proteasome inhibitors have previously been reported to cause apoptosis in cancer cells [13, 17, 32, 33]. Although the apoptotic effect of ixazomib on lung cancer cells has not been previously investigated, its apoptotic effects have been observed on many other solid tumour cell lines (colon, prostate, hepatocellular carcinoma cells) [17, 34, 35].

EMT is a process in which cells morphologically transition from the epithelial phenotype to the mesenchymal fibroblastoid phenotype and plays an important role in the cancer metastasis process [8]. TNF- α , released by M1 macrophages, is an important cytokine that modulates inflammatory responses in the microenvironment of many tumours [36]. Although studies have reported many factors, such as EGF, TGF- β , TNF- α , generally found in the tumour microenvironment, induce EMT [37], more recent studies have also reported that TNF- α inhibits EMT by suppressing TGF- β levels [38]. Furthermore, the TLR4/IL-10

axis has been implicated in the EMT signaling pathway, and inhibition of effectively suppresses EMT induction. [39]. In our results, decreased TNF- α and increased IL-10 levels in A549 cells and M2a and M2c polarised macrophage co-culture are responsible for increased EMT in A549 cells. Also, EMT was induced in A549 cancer cells in co-culture groups formed with M2a and M2c macrophages, depending on the expression levels of vimentin, E-cadherin, fibronectin, NF- κ B, VEGF and CCL-17 mRNA. An increase in TGF- β mRNA expression is a particularly relevant result because studies have shown that TGF- β is an important factor for EMT and can be an EMT-inducing agent [38, 40]. According to our results, TGF- β may play a role in the induction of EMT in A549 cells with co-culture of M2a and M2c macrophages.

Factors such as CCL-17 derived from tumour-associated macrophages are important contributors to immunosuppression. Studies have shown that this causes and intensifies various stages of cancer progression [41]. The high levels of CCL-17 mRNA expression levels found only in the M2c macrophage and A549 co-culture group are informative.

NF- κ B is a key transcriptional factor responsible for regulating the inflammatory signal and is important in cellular proliferation and differentiation. It's a key marker for cancer invasion and EMT, and it's involved in both the induction and support of EMT. As a result, it has been proposed that NF- κ B activity decreases the expression of genes associated with EMT [10, 11, 42]. We observed, bortezomib and ixazomib 'well-known NF- κ B inhibitors' decreased M2c-induced EMT.

E-cadherin is recognised as the main marker of epithelial morphology [43], and E-cadherin expression usually decreases during EMT, while the levels of mesenchymal specific marker vimentin increase [44]. We determined vimentin and E-cadherin mRNA expression and monoclonal antibody levels correlated to the increase in EMT in the A549 and M2c macrophage co-cultures were more significant than M2a macrophage groups.

Conclusion

M2c macrophages promote EMT in lung cancer cells, according to our findings. M1 macrophages, on the other hand, reduce the proliferation and viability of A549 cancer cells and induce apoptosis in those cells. The observed inhibition of M2c-induced EMT in A549 cells exposed to bortezomib and ixazomib has provided insight into an unknown aspect of proteasome inhibition of the EMT pathway, which is the current therapeutic approach. This is the first study to show that hydrocortisone polarizes M2c macrophages, to our knowledge. Our findings suggest that in lung cancer patients taking glucocorticoid-related drugs, the M1/M2 macrophage amount in the tumor microenvironment should be investigated. Finally, polarising tumour-associated macrophages to M1 and eliminating M2a or particularly M2c macrophages, after additional investigation, might represent effective future anti-cancer therapy strategies.

Declarations

Data availability: All data generated or analysed during this study are included in this published article (and its supplementary information files).

Compliance with Ethical Standards

Funding: This study was funded by Anadolu University Scientific Research Projects (grant number 1704S095.).

Conflict of interest: Author Selin Engür-Öztürk declares that she has no conflict of interest. Author Miriş Dikmen declares that she has no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

References

- [1] Siegel, R. L., Miller, K. D., & Jemal, A. (2019). Cancer statistics, 2019. *CA: a cancer journal for clinicians*, 69(1), 7–34. <https://doi.org/10.3322/caac.21551>.
- [2] Denisenko, T. V., Budkevich, I. N., & Zhivotovsky, B. (2018). Cell death-based treatment of lung adenocarcinoma. *Cell death & disease*, 9(2), 117. <https://doi.org/10.1038/s41419-017-0063-y>.
- [3] Yu, L., Li, K., & Zhang, X. (2017). Next-generation metabolomics in lung cancer diagnosis, treatment and precision medicine: mini review. *Oncotarget*, 8(70), 115774–115786. <https://doi.org/10.18632/oncotarget.22404>.
- [4] Tsai, M. J., Chang, W. A., Huang, M. S., & Kuo, P. L. (2014). Tumor microenvironment: a new treatment target for cancer. *ISRN biochemistry*, 2014, 351959. <https://doi.org/10.1155/2014/351959>.
- [5] Sousa, S., Brion, R., Lintunen, M., Kronqvist, P., Sandholm, J., Mönkkönen, J., Kellokumpu-Lehtinen, P. L., Lauttia, S., Tynnenen, O., Joensuu, H., Heymann, D., & Määttä, J. A. (2015). Human breast cancer cells educate macrophages toward the M2 activation status. *Breast cancer research : BCR*, 17(1), 101. <https://doi.org/10.1186/s13058-015-0621-0>.
- [6] Mosser, D. M., & Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology*, 8(12), 958–969. <https://doi.org/10.1038/nri2448>.
- [7] Martinez, F. O., Helming, L., & Gordon, S. (2009). Alternative activation of macrophages: an immunologic functional perspective. *Annual review of immunology*, 27, 451–483. <https://doi.org/10.1146/annurev.immunol.021908.132532>.
- [8] Gao, D., Vahdat, L. T., Wong, S., Chang, J. C., & Mittal, V. (2012). Microenvironmental regulation of epithelial-mesenchymal transitions in cancer. *Cancer research*, 72(19), 4883–4889. <https://doi.org/10.1158/0008-5472.CAN-12-1223>.

- [9] Micalizzi, D. S., Farabaugh, S. M., & Ford, H. L. (2010). Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. *Journal of mammary gland biology and neoplasia*, 15(2), 117–134. <https://doi.org/10.1007/s10911-010-9178-9>.
- [10] Podo, F., Buydens, L. M., Degani, H., Hilhorst, R., Klipp, E., Gribbestad, I. S., Van Huffel, S., van Laarhoven, H. W., Luts, J., Monleon, D., Postma, G. J., Schneiderhan-Marra, N., Santoro, F., Wouters, H., Russnes, H. G., Sørli, T., Tagliabue, E., Børresen-Dale, A. L., & FEMME Consortium (2010). Triple-negative breast cancer: present challenges and new perspectives. *Molecular oncology*, 4(3), 209–229. <https://doi.org/10.1016/j.molonc.2010.04.006>.
- [11] Hsu, Y. L., Chen, C. Y., Lin, I. P., Tsai, E. M., Kuo, P. L., & Hou, M. F. (2012). 4-Shogaol, an active constituent of dietary ginger, inhibits metastasis of MDA-MB-231 human breast adenocarcinoma cells by decreasing the repression of NF- κ B/Snail on RKIP. *Journal of agricultural and food chemistry*, 60(3), 852–861. <https://doi.org/10.1021/jf2052515>.
- [12] Shishodia, S., & Aggarwal, B. B. (2002). Nuclear factor-kappaB activation: a question of life or death. *Journal of biochemistry and molecular biology*, 35(1), 28–40. <https://doi.org/10.5483/bmbrep.2002.35.1.028>.
- [13] Engür, S., Dikmen, M., & Öztürk, Y. (2016). Comparison of antiproliferative and apoptotic effects of a novel proteasome inhibitor MLN2238 with bortezomib on K562 chronic myeloid leukemia cells. *Immunopharmacology and immunotoxicology*, 38(2), 87–97. <https://doi.org/10.3109/08923973.2015.1122616>.
- [14] Dikmen, M., Öztürk, S. E., Cantürk, Z., Ceylan, G., Karaduman, A. B., & Yamaç, M. (2020). Anticancer and antimetastatic activity of *Hypomyces chrysospermus*, a cosmopolitan parasite in different human cancer cells. *Molecular biology reports*, 47(5), 3765–3778. <https://doi.org/10.1007/s11033-020-05468-6>.
- [15] Mustafa, A. M., Maggi, F., Papa, F., Kaya, E., Dikmen, M., & Öztürk, Y. (2016). Isofuranodiene: A neuritogenic compound isolated from wild celery (*Smyrnum olusatrum* L., Apiaceae). *Food chemistry*, 192, 782–787. <https://doi.org/10.1016/j.foodchem.2015.07.079>.
- [16] Dikmen, M., Canturk, Z., Tilki, E. K., & Engur, S. (2017). Evaluation of antiangiogenic and antimetastatic Effects of *Penicillium chrysogenum* Secondary Metabolites. *Indian Journal of Pharmaceutical Sciences*, 79(1), 49-57. <https://doi.org/10.4172/pharmaceutical-sciences.1000200>.
- [17] Engür, S., & Dikmen, M. (2017). The evaluation of the anti-cancer activity of ixazomib on Caco2 colon solid tumor cells, comparison with bortezomib. *Acta clinica Belgica*, 72(6), 391–398. <https://doi.org/10.1080/17843286.2017.1302623>.
- [18] Mustafa, A. M., Caprioli, G., Dikmen, M., Kaya, E., Maggi, F., Sagratini, G., ... & Öztürk, Y. (2015). Evaluation of neuritogenic activity of cultivated, wild and commercial roots of *Gentiana lutea* L. *Journal of Functional Foods*, 19, 164-173. <https://doi.org/10.1016/j.jff.2015.09.018>.

- [19] Finnin, M., Hamilton, J. A., & Moss, S. T. (1999). Characterization of a CSF-induced proliferating subpopulation of human peripheral blood monocytes by surface marker expression and cytokine production. *Journal of leukocyte biology*, 66(6), 953–960. <https://doi.org/10.1002/jlb.66.6.953>.
- [20] Verreck, F. A., de Boer, T., Langenberg, D. M., van der Zanden, L., & Ottenhoff, T. H. (2006). Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-gamma- and CD40L-mediated costimulation. *Journal of leukocyte biology*, 79(2), 285–293. <https://doi.org/10.1189/jlb.0105015>.
- [21] Chanput, W., Mes, J. J., Savelkoul, H. F., & Wichers, H. J. (2013). Characterization of polarized THP-1 macrophages and polarizing ability of LPS and food compounds. *Food & function*, 4(2), 266–276. <https://doi.org/10.1039/c2fo30156c>.
- [22] Yeung, O. W., Lo, C. M., Ling, C. C., Qi, X., Geng, W., Li, C. X., Ng, K. T., Forbes, S. J., Guan, X. Y., Poon, R. T., Fan, S. T., & Man, K. (2015). Alternatively activated (M2) macrophages promote tumour growth and invasiveness in hepatocellular carcinoma. *Journal of hepatology*, 62(3), 607–616. <https://doi.org/10.1016/j.jhep.2014.10.029>.
- [23] Yuan, A., Hsiao, Y. J., Chen, H. Y., Chen, H. W., Ho, C. C., Chen, Y. Y., Liu, Y. C., Hong, T. H., Yu, S. L., Chen, J. J., & Yang, P. C. (2015). Opposite Effects of M1 and M2 Macrophage Subtypes on Lung Cancer Progression. *Scientific reports*, 5, 14273. <https://doi.org/10.1038/srep14273>.
- [24] Stein, M., Keshav, S., Harris, N., & Gordon, S. (1992). Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *The Journal of experimental medicine*, 176(1), 287–292. <https://doi.org/10.1084/jem.176.1.287>.
- [25] Varin, A., & Gordon, S. (2009). Alternative activation of macrophages: immune function and cellular biology. *Immunobiology*, 214(7), 630–641. <https://doi.org/10.1016/j.imbio.2008.11.009>.
- [26] Gordon, S., & Martinez, F. O. (2010). Alternative activation of macrophages: mechanism and functions. *Immunity*, 32(5), 593–604. <https://doi.org/10.1016/j.immuni.2010.05.007>.
- [27] Genin, M., Clement, F., Fattaccioli, A., Raes, M., & Michiels, C. (2015). M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. *BMC cancer*, 15, 577. <https://doi.org/10.1186/s12885-015-1546-9>.
- [28] Godugu, C., Patel, A. R., Desai, U., Andey, T., Sams, A., & Singh, M. (2013). AlgiMatrix™ based 3D cell culture system as an in-vitro tumor model for anticancer studies. *PloS one*, 8(1), e53708. <https://doi.org/10.1371/journal.pone.0053708>.
- [29] Forde, P. M., Kelly, R. J., & Brahmer, J. R. (2014). New strategies in lung cancer: translating immunotherapy into clinical practice. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 20(5), 1067–1073. <https://doi.org/10.1158/1078-0432.CCR-13-0731>.

- [30] Foey, A. D. (2014). Macrophages—masters of immune activation, suppression and deviation. Immune response activation. <https://doi.org/10.5772/57541>.
- [31] Huang, X., Li, Y., Fu, M., & Xin, H. B. (2018). Polarizing Macrophages In Vitro. *Methods in molecular biology* (Clifton, N.J.), 1784, 119–126. https://doi.org/10.1007/978-1-4939-7837-3_12.
- [32] Dutaud, D., Aubry, L., Henry, L., Levieux, D., Hendil, K. B., Kuehn, L., Bureau, J. P., & Ouali, A. (2002). Development and evaluation of a sandwich ELISA for quantification of the 20S proteasome in human plasma. *Journal of immunological methods*, 260(1-2), 183–193. [https://doi.org/10.1016/s0022-1759\(01\)00555-5](https://doi.org/10.1016/s0022-1759(01)00555-5).
- [33] Chauhan, D., Tian, Z., Zhou, B., Kuhn, D., Orlowski, R., Raje, N., Richardson, P., & Anderson, K. C. (2011). In vitro and in vivo selective antitumor activity of a novel orally bioavailable proteasome inhibitor MLN9708 against multiple myeloma cells. *Clinical cancer research: an official journal of the American Association for Cancer Research*, 17(16), 5311–5321. <https://doi.org/10.1158/1078-0432.CCR-11-0476>.
- [34] Wei, X., Zhou, P., Lin, X., Lin, Y., Wu, S., Diao, P., Xie, H., Xie, K., & Tang, P. (2014). MLN2238 synergizes BH3 mimetic ABT-263 in castration-resistant prostate cancer cells by induction of NOXA. *Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine*, 35(10), 10213–10221. <https://doi.org/10.1007/s13277-014-2333-y>.
- [35] Augello, G., Modica, M., Azzolina, A., Puleio, R., Cassata, G., Emma, M. R., Di Sano, C., Cusimano, A., Montalto, G., & Cervello, M. (2018). Preclinical evaluation of antitumor activity of the proteasome inhibitor MLN2238 (ixazomib) in hepatocellular carcinoma cells. *Cell death & disease*, 9(2), 28. <https://doi.org/10.1038/s41419-017-0195-0>.
- [36] Van Ginderachter, J. A., Movahedi, K., Hassanzadeh Ghassabeh, G., Meerschaut, S., Beschin, A., Raes, G., & De Baetselier, P. (2006). Classical and alternative activation of mononuclear phagocytes: picking the best of both worlds for tumor promotion. *Immunobiology*, 211(6-8), 487–501. <https://doi.org/10.1016/j.imbio.2006.06.002>.
- [37] Wang, Y., Shi, J., Chai, K., Ying, X., & Zhou, B. P. (2013). The Role of Snail in EMT and Tumorigenesis. *Current cancer drug targets*, 13(9), 963–972. <https://doi.org/10.2174/15680096113136660102>.
- [38] Dash, S., Sarashetti, P. M., Rajashekar, B., Chowdhury, R., & Mukherjee, S. (2018). TGF- β 2-induced EMT is dampened by inhibition of autophagy and TNF- α treatment. *Oncotarget*, 9(5), 6433–6449. <https://doi.org/10.18632/oncotarget.23942>.
- [39] Suarez-Carmona, M., Lesage, J., Cataldo, D., & Gilles, C. (2017). EMT and inflammation: inseparable actors of cancer progression. *Molecular oncology*, 11(7), 805–823. <https://doi.org/10.1002/1878-0261.12095>.

- [40] Yang, Y., Luo, N. S., Ying, R., Xie, Y., Chen, J. Y., Wang, X. Q., Gu, Z. J., Mai, J. T., Liu, W. H., Wu, M. X., Chen, Z. T., Fang, Y. B., Zhang, H. F., Zuo, Z. Y., Wang, J. F., & Chen, Y. X. (2017). Macrophage-derived foam cells impair endothelial barrier function by inducing endothelial-mesenchymal transition via CCL-4. *International journal of molecular medicine*, 40(2), 558–568. <https://doi.org/10.3892/ijmm.2017.3034>
- [41] Sawa-Wejksza, K., & Kandefer-Szerszeń, M. (2018). Tumor-Associated Macrophages as Target for Antitumor Therapy. *Archivum immunologiae et therapiae experimentalis*, 66(2), 97–111. <https://doi.org/10.1007/s00005-017-0480-8>.
- [42] Huber, M. A., Azoitei, N., Baumann, B., Grünert, S., Sommer, A., Pehamberger, H., Kraut, N., Beug, H., & Wirth, T. (2004). NF-kappaB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. *The Journal of clinical investigation*, 114(4), 569–581. <https://doi.org/10.1172/JCI21358>.
- [43] Tsai, J. H., & Yang, J. (2013). Epithelial-mesenchymal plasticity in carcinoma metastasis. *Genes & development*, 27(20), 2192–2206. <https://doi.org/10.1101/gad.225334.113>.
- [44] Varga, J., De Oliveira, T., & Greten, F. R. (2014). The architect who never sleeps: tumor-induced plasticity. *FEBS letters*, 588(15), 2422–2427. <https://doi.org/10.1016/j.febslet.2014.06.019>.

Figures

Figure 1

M1, M2a and M2c macrophage polarization. (A1) Light microscope image of THP-1, THP-1 origin macrophage (M0), M1, M2a and M2c polarized macrophage cells (10X) (B1) Validation of M1, M2a/c polarization by the flow cytometry. CD80, CD23 and CD163 are specific surface markers of M1, M2a and M2c macrophages, respectively, and the marker CD206 is common to both M2 subtypes. Experiments were performed in triplicate (C1) Expressions of some M1/M2 markers in differentiated macrophage

subtypes, determined by RT-PCR. The gene expression of differentiated macrophage subtypes was normalized to that of untreated M0 macrophages. $p<0.05^*$, $p<0.01^{**}$, $p<0.0001^{***}$, (mean $\pm$ SD, $n = 3$).

Figure 2

Anti-proliferative effects of bortezomib and ixazomib on cells. (A2) A549 cell proliferation curves at 48 hours of bortezomib concentrations, IC_{50} : 45 nM (B2) A549 cell proliferation curves at 48 hours of ixazomib concentrations, IC_{50} : 2 μ M (C2) CCD-19 Lu cell proliferation curves at 48 hours of bortezomib concentrations, IC_{50} : 160 nM. (D2) CCD-19 Lu cell proliferation curves at 48 hours of ixazomib concentrations, IC_{50} : 10 μ M. (Arrow: application of concentration point, mean $\pm$ SD, n = 6). (E2) Concentrations of bortezomib and ixazomib in M0 macrophage cells and statistical assessment of the 24 and 48-hour percent viability values calculated using the WST-1 method. $p < 0.01^{**}$, $p < 0.0001^{****}$, (mean $\pm$ SD, n = 8).

Figure 3

Antiproliferative and apoptotic effects of macrophages on A549 cells. (A₃) Proliferation curves and slope graph of co-culture models created with A549 and M1, M2a and M2c polarized macrophage cells using an E-plate insert, determined in a real-time cell analysis system (Arrow: merge of A549 and macrophages, mean $\pm$ SD, n = 6, $p < 0.05^*$, $p < 0.0001^{****}$). **(B₃, C₃, D₃)** Proliferation curves and slope graph of bortezomib and ixazomib IC₅₀ concentrations in the A549 and M1, M2a or M2c polarized macrophage co-

culture model (Arrow: merge of A549 and macrophages and application of concentration point, mean $\pm$ SD, n = 6, $p < 0.01^{**}$, $p < 0.001^{***}$, $p < 0.0001^{****}$) **(E3)** Apoptosis of A549 cells and co-culture macrophage with or without drugs. Cell apoptosis was determined by flow cytometry with annexin V/PI-staining. Data were confirmed in three independent experiments (mean $\pm$ standard deviation, $p > 0.05$, $p < 0.0001^{****}$). **(F3)** Caspase-3 activity A549 cells and co-culture macrophage with or without drugs determined by flow cytometry. Data were confirmed in three independent experiments and the error bars shows the standard deviation ($p > 0.05$, $p < 0.05^{*}$, $p < 0.001^{***}$, $p < 0.0001^{****}$). **(G3)** Mitochondrial membrane depolarization of A549 cells was determined by flow cytometry with JC-1 staining. Data were confirmed in three independent experiments and the error bars shows the standard deviation ($p < 0.0001^{****}$).

Figure 4

M2c macrophages induced EMT. (A4) TNF- α and IL-10 cytokine levels (percentage) evaluated by flow cytometry in A549 cells and M1, M2a and M2c polarized macrophage co-culture groups (n=3, mean $\pm$ SD, $p < 0.01^{**}$, $p < 0.0001^{****}$). **(B4, D4)** Expressions of some EMT markers in A549 cells co-cultured with M1, M2a and M2c polarized macrophage with or without drugs, determined by RT-PCR. $p < 0.05^{*}$, $p < 0.01^{**}$, $p < 0.0001^{****}$, (mean $\pm$ SD, n = 3). **(C4)** Vimentin and E-cadherin levels of A549 cells was determined by

flow cytometry. In addition, the results of percent change and the statistical evaluation of vimentin and E-cadherin levels are shown in the graphic. Data were confirmed in three independent experiments and the error bars shows the standard deviation ($p<0.05^*$, $p<0.01^{**}$, $p<0.0001^{****}$). **(E4)** Morphological image of vimentin (red, Alexa Fluor 594) and E-cadherin (green, Alexa Fluor 488) in A549 cells undergoing immunofluorescence staining. Cell nuclei stained blue (with Hoechst 33258). The data expressed as the mean values $\pm$ SD ($p \leq 0.001$). Images were taken by Cytation 3 Cell Imaging Multi-Mode Reader with 10 $\times$ objective.

Figure 5

3D culture studies. (A5) Morphological imaging of spheroids in A549 lung cancer cells. Also, spheroid formations of A549 cells were imaged as fluorescence in Cytation 3 Cell Imaging Multi-Mode Reader with hoechst 33258 on day 7 (10X). **(B5)** Spheroid sizes obtained from cell images of A549 cells taken on different days ($n = 25$, mean $\pm$ standard deviation, $p<0.0001^{****}$) Random photos were taken for the

analysis of the areas of the spheroids. A total of 25 photographs were selected among these photographs for each day, including 5 photographs with at least 5 spheroids.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [certificate1309644.pdf](#)