Selective upregulation of TNFα expression in classically-activated human monocyte-derived macrophages (M1) through pharmacological interference with V-ATPase

Lea Thomas[a], Zhigang Rao[a], Jana Gerstmeier[a], Martin Raasch[b], Christina Weinigel[c], Silke Rummler[c], Dirk Menche[d], Rolf Müller[e], Carlo Pergola[a], Alexander Mosig[b], Oliver Werz[a,*]

[a]Chair of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich Schiller University Jena, Philosophenweg 14, 07743 Jena, Germany; E-mail addresses: lea.thomas@uni-jena.de; pergolacarlo@googlemail.com; rao.zhigang@uni-jena.de; jana.gerstmeier@uni-jena.de; oliver.werz@uni-jena.de

[b]Institute of Biochemistry II and Center for Sepsis Control and Care and [c]Institute of Transfusion Medicine, University Hospital Jena, Jena, Germany; E-mail addresses: christina.weinigel@med.uni-jena.de; silke.rummler@med.uni-jena.de; Alexander.MOSIG@med.uni-jena.de; Martin.Raasch@med.uni-jena.de

[d]Kekulé-Institut für Organische Chemie und Biochemie der Rheinischen Friedrich-Wilhelms-Universität Bonn, Bonn, Germany; E-Mail address: dirk.menche@uni-bonn.de

[e]Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Centre for Infection Research and Pharmaceutical Biotechnology at Saarland University; E-Mail address: Rolf.Mueller@helmholtz-hzi.de

* **Corresponding author:** Prof. Dr. Oliver Werz, Chair of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich-Schiller-University Jena, Philosophenweg 14, D-07743 Jena, Germany; Phone: +49-03641-949801; Fax: +49-03641-949802; e-mail: oliver.werz@uni-jena.de.

Abstract

Pharmacological interference with vacuolar-type H(+)-ATPase (V-ATPase), a proton-translocating enzyme involved in protein transport and pH regulation of cell organelles, is considered a potential strategy for cancer therapy. Macrophages are critically involved in tumor progression and may occur as pro-tumoral M2 phenotype, whereas classically-activated M1 can inhibit tumor development for example by releasing tumor-suppressing molecules, including tumor necrosis factor (TNF)α. Here, we show that targeting V-ATPase by selective inhibitors such as archazolid upregulates the expression and secretion of TNFα in lipopolysaccharide (LPS)- or LPS/interferon (INF)γ-activated M1-like macrophages derived from human blood monocytes. In contrast, archazolid failed to elevate TNFα production from uncommitted (M0) or interleukin (IL)-4-treated M2-like macrophages. Secretion of other relevant cytokines (i.e., IL-1β, IL-6, IL-10) or chemokines (i.e. IL-8 and monocyte chemotactic protein-1) from M1 was not affected by archazolid. Though V-ATPase inhibitors elevated the lysosomal pH in M1 comparable to chloroquine or ammonium chloride, the latter agents suppressed TNFα secretion. Archazolid selectively increased TNFα mRNA levels, which was abolished by dexamethasone. Interestingly, archazolid enhanced the phosphorylation and nuclear translocation of the p65 subunit of NFκB and stimulated phosphorylation of SAPK/JNK. In a microfluidically-supported human tumor biochip model, archazolid-treated M1 significantly reduced tumor cell viability. Together, our data show that V-ATPase inhibition selectively upregulates TNFα production in classically-activated macrophages along with NFκB and SAPK/JNK activation. Such increased TNFα release caused by V-ATPase inhibitors may contribute to tumor suppression in addition to direct targeting cancer cells.

Key words: vacuolar-type H(+)-ATPase, archazolid, tumor necrosis factor α, macrophages, interleukin, NFκB

Abbreviations used: FITC, fluorescein isothiocyanate; IL, interleukin; IFNγ, interferon γ; IκB inhibitor of nuclear factor kappa B; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; M-CSF, macrophage colony-stimulating factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NFκB, nuclear factor kappa B; p38 MAPK, p38 mitogen-activated protein kinase; PBMC, peripheral blood mononuclear cells; SAPK/JNK, stress-activated protein kinase/Jun amino-terminal kinases; TAM, tumor-associated macrophage; TNFα, tumor necrosis factorα; V-ATPase, vacuolar-type H(+)-ATPase

1. Introduction

Combination of direct cytotoxic activity against tumor cells with concomitant capacity to favorably modify the tumor microenvironment might be an appealing option for pharmacological treatment of diverse cancers with clinical relevance [1]. For example, the clinically used chemotherapeutic agent trabectedin, in addition to killing cancer cells, depletes monocytes and tumor-associated macrophages (TAMs) in tumor patients as a key component of its anti-tumor activity [2]. However, the discovery of relevant targets in this respect is still in demand in order to simultaneously repress the viability of cancer cells and to modulate tumor-associated mononuclear cells by a single agent.

The vacuolar-type H(+)-ATPases (V-ATPases) are ATP-dependent proton translocating macromolecular complexes that acidify lysosomes, endosomes, Golgi apparatus, and certain secretory granules in all eukaryotes [3, 4]. V-ATPases participate in physiological processes such as cellular pH homeostasis, receptor-mediated endocytosis, virus and toxin entry, intracellular trafficking as well as protein degradation and processing [3]. Aberrant regulation of pH by V-ATPases is implicated in several diseases including osteoporosis, renal tubular acidosis, and malignant neoplasms [5, 6]. V-ATPase is highly expressed on the plasma membrane of tumor cells and particularly contributes to the acidification of tumor microenvironments, thus supporting tissue damage, acquisition of metastatic cell phenotypes, and tumor invasiveness [6, 7]. Accordingly, specific inhibitors of V-ATPase that decrease the acidity of tumors, reduced the survival of tumor cells and tumor metastasis, and impaired chemoresistance [6, 8]. In fact, V-ATPase is considered as promising target in various types of cancer [6].

Cytokines and chemokines significantly impact the tumor microenvironment to which TAMs are exposed, and represent essential factors that impact the heterogeneity of TAM functions [9]. TAMs consist of different phenotypes with partly opposite impact on tumors: the M2-like subtypes play

pivotal roles in tumor progression by promoting cancer cell survival, angiogenesis, and immunosuppression, and they represent - as major part of TAMs - a remarkable fraction of tumor-infiltrating immune cells [9, 10]. In contrast, classically-activated M1-like subsets are indispensable innate immune cells with microbicidal and tumoricidal activity that produce high levels of anti-tumoral molecules such as tumor necrosis factor (TNF)α, thus opposing cancer cells and preventing the establishment and progression of cancers [1, 10].

The role of V-ATPase in cytokine secretion was studied in peritoneal macrophages from mice or rabbits before [11-13]. Here, we aimed to obtain insights into the role of V-ATPase in cytokine and chemokine secretion from human macrophages derived from peripheral blood monocytes, taking into account macrophage plasticity and occurrence as different phenotypes. As a suitable tool to studying the role of V-ATPase we used the myxobacterial compound archazolid [14] that binds to V-ATPase [15-17]. Archazolid has been intensively investigated as anti-tumoral agent that induced apoptosis of cancer cells and reduced migration of invasive tumor cells *in vitro*, and decreased metastatic dissemination of breast tumors *in vivo* [18-20]. In various highly invasive tumor cell lines and leukemic cells, archazolid led to apoptosis due to activation of the hypoxia-inducible factor-1α (HIF1α) [18, 21], and to anoikis induction [22]. Thus, archazolid represents a promising candidate for direct targeting of tumor cells via V-ATPase inhibition. We recently reported that archazolid reduces the secretion of cytokines and chemokines in primary human monocytes due to accumulation at the endoplasmic reticulum [23]. However, effects of archazolid on macrophages have not been addressed, and phenotype-specific functions of V-ATPase in cytokine/chemokine secretion from human macrophages have not been reported yet.

2. Materials and Methods

2.1. Materials

Archazolid B was isolated from *Archangium gephyra* as previously described [14]. Apicularen A was from the natural compound library of HZI/HIPS (Braunschweig, Germany). RPMI 1640 with L-glutamine, penicillin, streptomycin, and fetal calf serum (FCS) were from PAA Laboratories (Pasching, Germany). For in-house made ELISA, the capture and detection antibodies were from R&D Systems (Abington, UK). U0126, SB203580, nigericin, and SP600125 were from Enzo Life Sciences (Lörrach, Germany), LY294002 from BIOZOL Diagnostica Vertrieb GmbH (Eching, Germany), actinomycin D from Cayman Chemical (Ann Arbor, MI), staurosporine from Calbiochem (Merck, Darmstadt, Germany), interferon (INF)γ and interleukin (IL)-4 from PeproTech (Hamburg, Germany), macrophage colony-stimulating factor (M-CSF) from Cell Guidance Systems Ltd (Cambridge, UK), Calcein-AM and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Thermo Fisher Scientific Inc. (Waltham, MA), LysoTracker dye from Life Technologies (Schwerte, Germany) and ammonium chloride, bafilomycin A1, chloroquine, dexamethasone, diphenyleneiodonium chloride (DPI), LPS, parthenolide, propidium iodide and all other fine chemicals were from Sigma-Aldrich (Steinheim, Germany), unless indicated otherwise.

2.2. Isolation of monocytes, differentiation to macrophages and macrophage activation

The protocols for experiments with human monocytes were approved by the ethical commission of the Friedrich-Schiller-University Jena. All methods were performed in accordance with the relevant guidelines and regulations. Leukocyte concentrates from peripheral blood of healthy human donors which did not take anti-inflammatory medication for the last ten days were obtained from the Institute of Transfusion Medicine, University Hospital Jena, and prepared as described [23]. Peripheral blood

mononuclear cells (PBMC) were isolated by dextran sedimentation and density centrifugation on lymphocyte separation medium (LSM 1077, PAA Laboratories, Pasching, Austria). Monocytes from the PBMC fraction were isolated by adherence for 1 hr at 37 °C and 5% CO_2 to culture flasks (2×10^7 cells/mL RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin) [24].

For differentiation towards macrophages, freshly isolated monocytes were incubated for 6 days in RPMI 1640 supplemented with 5% FCS and human recombinant M-CSF (25 ng/mL) [25]. To obtain classically-activated macrophages (M1-like), cells were further treated with 100 ng/mL LPS (designated M_{LPS}) or 100 ng/mL LPS plus 100 ng/mL human recombinant IFNγ (M1) for 24 hrs. To obtain M2-like cells, macrophages were incubated with human recombinant IL-4 (20 ng/mL) for 24 hrs [25, 26]. To assure correct polarization, cells were assessed by flow cytometry for expression of the M2 surface markers CD163 and CD206, while M1 and M_{LPS} were characterized by expression of CD40 and higher expression of HLA-DR (MHC class II receptor) than M2 [26].

2.3. Determination of cell viability

The viability of human macrophages was assessed by MTT assay as described [27]. Briefly, macrophages after 6 days of differentiation of monocytes were pre-incubated with compounds for 30 min at 37 °C (5% CO_2) and treated for 24 hrs with (I) 100 ng/mL LPS (M_{LPS}), (II) 100 ng/mL LPS plus 100 ng/mL INFγ (M1), (III) 20 ng/mL IL-4 (M2) or left untreated (M0). MTT was added, cells were further incubated for 4 hrs, and lysed in a buffer containing 10% (w/v) SDS. Staurosporine, a pan-kinase inhibitor and inducer of apoptosis, was used as positive control.

2.4. Immunofluorescence microscopy and live cell imaging

Macrophages (2.5×10^5/mL) were seeded on glass coverslips and incubated for 1 hr at 37 °C and 5% CO_2. After attachment, cells were pre-incubated with test compounds (as indicated) and stimulated with LPS (100 ng/mL), with LPS (100 ng/mL) plus IFNγ (100 ng/mL) or with IL-4 (20 ng/mL) for the indicated times at 37 °C and 5% CO_2. Then, cells were fixed with 4% (v/v) paraformaldehyde solution, autofluorescence of free formaldehyde was quenched with 50 mM ammonium chloride, and macrophages were permeabilized with 0.2% (v/v) Triton X-100. After blocking with 10% (v/v) non-immune goat serum (Invitrogen, Darmstadt, Germany), samples were incubated with antibodies against the c-subunit of V-ATPase (1:1000 anti-ductin, Biozol, Eching, Germany), LAMP-1 (1:1000, Abcam, Cambridge, UK), IκBα (1:125, mouse monoclonal anti-IκBα, Cell Signaling Technology, Danvers, MA) and NFκB (1:100, rabbit monoclonal anti-NFκB p65, Cell Signaling Technology) over night at 4 °C. The samples were washed and then stained with the fluorophore-labeled secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG (1:500) and Alexa Fluor 555 goat anti-mouse IgG (1:500) (Invitrogen) for 10 min at RT in the dark. DNA was stained using DAPI (0.7 µg/mL) for 3 min at RT. Samples were placed on microscope slides (Roth, Karlsruhe, Germany) using Mowiol containing 0.25% *n*-propyl gallate (Sigma-Aldrich). The fluorescence was visualized with a Zeiss Axio Observer.Z1 inverted microscope (Carl Zeiss, Jena, Germany) and a LCI Plan-Neofluar 63x/1.3 Imm Corr DIC M27 objective. Images were taken with an AxioCam MR3 camera and were acquired, cut, linearly adjusted in the overall brightness and contrast, and exported by the AxioVision 4.8 software.

For imaging of living cells, macrophages (2.5×10^5/mL) were plated into glass bottom dishes (MatTek Corporation, Ashland, MA). After adherence, cells were treated with archazolid (100 nM) or vehicle for 30 min and stimulated for 24 hrs with either (I) 100 ng/mL LPS, (II) 100 ng/mL LPS plus 100 ng/mL INFγ, or (III) 20 ng/mL IL-4.

For imaging of fluorescein isothiocyanate (FITC) dextran (Thermo Fisher Scientific Inc.) uptake into macrophages, cells were treated as described in pH measurements. Cells were imaged immediately using the Axio Observer Z1 inverted microscope and a Plan-Apochromat 40x/1,3 Oil DIC M27 objective or a LCI Plan-Neofluar 63x/1.3 Imm Corr DIC M27 objective.

2.5. Determination of cytokine/chemokine levels

After 6 days of differentiation of monocytes to macrophages with M-CSF (25 ng/mL) at 37 °C and 5% CO_2, test compounds or vehicle were added to macrophages and 30 min later, cells were incubated for 24 hrs with either (I) 100 ng/mL LPS, (II) 100 ng/mL LPS plus 100 ng/mL INFγ, or (III) 20 ng/mL IL-4. For measurement of extracellular cytokine levels, supernatants were collected by centrifugation (2000×*g*, 4 °C, 10 min). Cytokines and chemokines were either analyzed by using commercially available ELISA kits (IL-8, Assaypro, St. Charles, MO) according to manufacturer´s specifications, or by in-house made ELISA (IL-1β, IL-6, IL-10, TNFα, monocyte chemotactic protein (MCP)-1).

2.6. pH measurements in macrophages

pH measurements were performed according to [28], and compositions of sodium and potassium buffers were used as described [29]. For measurements of the vesicular pH, macrophages were incubated with 0.5 mg/mL FITC-dextran (70,000 MW, Sigma-Aldrich) for 1 hr. Cells were washed with PBS, pre-incubated with test compounds or vehicle for 30 min and stimulated with 100 ng/mL LPS for 24 hrs. The pH values were calculated from the ratio of fluorescence intensities excited at 480 nm and 450 nm and emission at 520 nm. *In situ* fluorescence calibration was performed with the ionophore nigericin (10 μM) in potassium buffer at pH values between 4.5 and 7.5.

For analysis by LysoTracker® staining, macrophages (2.5×10^5/mL) were plated into glass bottom

dishes (MatTek Corporation). After adherence, cells were treated with test compounds or vehicle for 30 min and stimulated with 100 ng/mL LPS for 24 hrs. Macrophages were then stained with the fluorescent LysoTracker® dye (50 nM) for 1 hr. After washing, the red fluorescence of the accumulated probe in acidic cell organelles was imaged using the Axio Observer Z1 microscope and a LCI Plan-Neofluar 63x/1.3 Imm Corr DIC M27 objective.

2.7. Relative quantification of TNFα and IL-1β mRNA levels in macrophages and determination of TNFα mRNA degradation

After 6 days of differentiation of monocytes to macrophages with M-CSF (25 ng/mL), macrophages were pre-incubated with test compounds or vehicle for 30 min and stimulated with 100 ng/mL LPS for the indicated times at 37 °C and 5% CO_2. For measurement of TNFα mRNA degradation, macrophages were stimulated with 100 ng/mL LPS for 24 hrs and incubated with archazolid or vehicle for 1 hr. Then, transcription was blocked by addition of actinomycin D (5 µg/mL) and cells were incubated for the indicated times. Total RNA was isolated using the E.Z.N.A.® Total RNA Kit I (Omega Biotek, Norcross, GA) and cDNA was generated by reverse transcription with SuperScript® III First-Strand Synthesis SuperMix (Invitrogen). cDNA was amplified by PCR and quantified using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Darmstadt, Germany). To calculate the relative TNFα mRNA expression (normalized to B2M) the $2^{(-\Delta\Delta C(T))}$ method was applied [30]. Primers used: human beta-2-microglobulin (B2M) forward primer 5´-CTCCGTGGCCTTAGCTGTG -3´, human B2M reverse primer 5´-TTTGGAGTACGCTGGATAGCCT -3´, human TNFα forward primer 5'-CCCAGGGACCTCTCTCTAATC-3', human TNFα reverse primer 5'-ATGGGCTACAGGCTTGTCACT-3', human IL-1β forward primer 5'-ACAGATGAAGTGCTCCTTCCA-3', human IL-1β reverse primer 5'-GTCGGAGATTCGTAGCTGGAT-3' (Tib Molbiol, Berlin, Germany).

2.8. Determination of phosphorylation of protein kinases and transcription factors by Western blot analysis

Macrophages, obtained by differentiation of monocytes with M-CSF (25 ng/mL) at 37 °C and 5% CO_2 for 6 days, were first starved for 6 hrs (AKT, ERK-1/2, p38 MAPK, MEK1/2, SAPK/JNK, MKK3/6) or directly pre-incubated (NFκB p65, STAT-1, STAT-3) with archazolid (100 nM) or vehicle (0.1% DMSO) for 30 min at 37 °C, 5% FCS was added and cells were stimulated with LPS (100 ng/mL) for 15 min. Macrophages were placed on ice, washed once with PBS and lysed with a NP-40 lysis buffer (1% (v/v) NP-40, 1 mM sodium vanadate, 10 mM sodium fluoride, 5 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 5 mM EDTA, and freshly added 10 µg/mL leupeptin hemisulfate salt, 60 µg/mL trypsin inhibitor from soybean, 1 mM phenylmethylsulfonyl fluoride in TBS, pH 7.4). Lysates were centrifuged (10,000×*g*, 4 °C, 5 min), and protein concentrations in the supernatants were determined using a Protein Assay (Bio-Rad Laboratories, Hercules, CA). After addition of 4×SDS loading buffer (50 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) β-mercaptoethanol, 12.5 mM EDTA, 0.02% (w/v) bromophenol blue) to the lysates, samples were boiled for 5 min at 95 °C. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK). After blocking with 5% (w/v) bovine serum albumin or low-fat milk powder (Roth, Karlsruhe, Germany), membranes were incubated with primary antibodies overnight at 4 °C. Antibodies, obtained from Cell Signaling Technology (Danvers, MA), were used as follows: NFκB p65, 1:500, rabbit monoclonal anti-NFκB p65; phospho-NFκB p65, 1:500, mouse monoclonal anti-phospho-NFκB p65; phospho-STAT-1, 1:1000, rabbit polyclonal anti-phospho-STAT-1; phospho-STAT-3, 1:1000, rabbit monoclonal anti-phospho-STAT-3; p38 MAPK, 1:1000, rabbit monoclonal anti-p38 MAPK; phospho-p38 MAPK, 1:1000, rabbit polyclonal anti-phospho-p38 MAPK; ERK-1/2, 1:1000, rabbit polyclonal anti-p44/42 ERK; phospho-ERK-1/2, 1:1000, mouse monoclonal anti-phospho-p44/42 ERK; Akt, 1:1000, mouse monoclonal anti-Akt;

phospho-Akt, 1:1000, rabbit polyclonal anti-phospho-Akt; MEK1/2, 1:1000, rabbit polyclonal anti-MEK1/2; phospho-MEK1/2, 1:1000, rabbit polyclonal anti-phospho-MEK1/2; phospho-MKK3, 1:1000, rabbit monoclonal anti-phospho-MKK3/6; phospho-SAPK/JNK, 1:1000, mouse monoclonal anti-phospho-SAPK/JNK. Antibodies against GAPDH: 1:1000, mouse monoclonal anti-GAPDH (Santa Cruz Biotechnology, Heidelberg, Germany) and β-actin, 1:1000, mouse monoclonal anti-β-actin (Santa Cruz Biotechnology). Membranes were washed and incubated with fluorescently-labeled secondary antibodies (LI-COR Biosciences, Bad Homburg, Germany) for 1 hr at RT in the dark. Proteins were detected using the Odyssey imaging system (LI-COR Biosciences, Bad Homburg, Germany).

2.9. Measurement of ROS formation

ROS levels were assessed as reported before [31]. Briefly, macrophages were incubated with test compounds or vehicle (0.1% DMSO) for 30 min and stimulated with LPS (100 ng/mL) for 24 hrs or left untreated. Cells were washed and incubated in HBSS buffer containing 2'-7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich) (10 μM) for 30 min at 37 °C/5% CO_2. To detect and quantify intracellular formation of ROS, the fluorescence of the deacetylated dye (2'-7'-dichlorofluorescein) was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a NOVOstar microplate reader (BMG Labtechnologies GmbH, Offenburg, Germany).

2.10. Microfluidically supported biochip assay

Biochips were prepared by injection moulding from cyclo olefin polymer (COP) Zeonor®, obtained from microfluidic ChipShop GmbH (Jena, Germany), and manufactured as described previously [32]. MCF-7 cells were obtained at LGC Standards (Wesel, Germany). Authentication of MCF-7 was carried out by STR analyses (LGC Standards) and experiments were completed within 4 months of

receipt of the cell line. Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords and cultured for up to four passages as described previously [32]. Donors were informed about the aim of the study and gave written consent. HUVEC were seeded at top of the biochip-embedded membrane, MCF-7 cells were seeded at the lower compartment of the biochip. Monocytes (in a six well plate) were differentiated towards macrophages in presence of M-CSF for 6 days, and M1 polarization was induced by addition of LPS plus IFNγ while M2 polarization was induced by addition of IL-4 for 24 hrs. These M1 and M2 were then added to the HUVEC into the biochip. After 24 hrs co-culture with HUVECs, medium containing 30 nM archazolid or vehicle (0.1% DMSO) was added, and after 30 min, it was replaced by fresh medium (without archazolid). Then, cells perfused over the vascular layer with a shear stress rate of 3 dyn/cm² for 30 min, followed by 24 hrs incubation with medium. In control experiments, macrophages were omitted and only HUVECs were used in the upper compartment. The viability of the cells was analyzed for by immunofluorescence microscopy using Calcein-AM (viable cells, green) and propidium iodide (dead cells, red) staining. Mean fluorescence intensity (MFI) of the Calcein-AM staining was measured by random field analysis of 40 regions of interest per experiment.

2.11. Statistics

Data are presented as mean + standard error of the mean (SEM) of *n* experiments, where *n* represents the number of experiments. Statistical data were calculated using GraphPad InStat program (GraphPad Software, CA), and were analyzed by Student's *t* test for paired groups and by one-way ANOVA for independent or correlated samples followed by a Bonferroni (< 5 groups) or Tukey-Kramer (> 5 groups) post hoc test for multiple comparisons. A P value of < 0.05 (*) was considered significant.

3. Results

3.1. Expression of V-ATPase in macrophages and effects of the V-ATPase inhibitor archazolid on the viability and morphology of macrophages.

According to well-established protocols, we induced M-CSF-treated macrophages (designated M0) with LPS/INFγ towards classically-activated M1 or with IL-4 towards M2-like subsets [33]. Moreover, M-CSF-treated macrophages were activated by LPS alone (designated M_{LPS}), which strongly resemble M1 [26] similar to macrophages activated by INFγ alone [33]. Previous studies showed that macrophages from different origins and species express V-ATPase and respond to V-ATPase inhibitors in different ways [13, 31, 34-39]. We first confirmed V-ATPase expression in human monocyte-derived macrophages [40] by Western blot (not shown) and by using immunofluorescence microscopy where no obvious differences regarding V-ATPase subcellular localization between the macrophage phenotypes were immediately apparent (**Fig. 1A**). Side-by-side analysis of LAMP-1, a marker protein for lysosomes, revealed colocalisation with V-ATPase (merge, **Fig. 1B**), suggesting that V-ATPase might be located to vesicles.

The V-ATPase inhibitor archazolid effectively induces apoptosis in cancer cells leading to cell death [18, 19]. Since we aimed at using arachzolid as tool compound to study V-ATPase in various human macrophage phenotypes, we first analyzed whether archazolid may affect the viability of macrophages. Incubation of activated macrophages (M_{LPS}, M1 or M2) with archazolid (1, 10, or 100 nM) revealed no loss of cell viability within 24 hrs, whereas the pan-protein kinase inhibitor and apoptosis inducer staurosporine (3 μM) efficiently reduced cell viability, as expected (**Fig. 1C**). Microscopic analysis confirmed cellular integrity of M_{LPS}, M1, and M2 upon archazolid treatment but revealed obvious alterations of the cell shape for M_{LPS} and M1 that was not evident for M2, for which the morphology was seemingly not affected by archazolid (**Fig. 1D**).

3.2. V-ATPase inhibitors induce TNFα secretion in classically-activated macrophages

Having confirmed that archazolid is not detrimental for cellular integrity and viability of macrophages, we next tested whether the compound could modulate cytokine and chemokine release. Differentiated macrophages (M0) were pretreated with archazolid and then either activated by LPS/INFγ (M1) or IL-4 (M2) for 24 hrs. Analysis of IL-1β, IL-6, IL-8, IL-10 and MCP-1 in the medium revealed no significant modulation of the release of these proteins by archazolid neither in M1 nor in M2 (**Fig. 2A**). However, the levels of TNFα were upregulated by archazolid (100 nM) about 2.4-fold in M1, whereas the compound failed to alter TNFα levels in M2 (**Fig. 2A**) and in M0 (not shown). More detailed concentration response experiments showed that for M_{LPS} or M1 the maximal effect (2.9- to 3.4-fold) on TNFα release was obtained at 30 nM archazolid and slightly declined at 100 nM (**Fig. 2B**). In contrast to archazolid and as expected, dexamethasone repressed TNFα levels in M_{LPS} and M1 (**Fig. 2B**). To confirm that the observed effect of archazolid is related to interference with V-ATPase, we tested other V-ATPase inhibitors, that is, apicularen A and bafilomycin A1 (1, 10 and 100 nM, each) in the same experimental settings. For both compounds upregulation of TNFα levels was evident in M_{LPS} and M1 with maximal effects at 10 nM (**Fig. 2C**), supporting that suppression of V-ATPase accounts for increased TNFα release.

3.3. Archazolid causes elevation of the lysosomal pH in classically-activated macrophages

Blocking V-ATPase is well-known to cause an elevated lysosomal pH in various cell types [16, 41, 42], and along these lines, archazolid led to significantly increased lysosomal pH in M_{LPS} as evidenced by the pH-dependent internalization of FITC-labeled dextran (**Fig. 3A**). Elevation of the lysosomal pH by archazolid was pronounced at 100 nM but only slightly affected at 10 nM. In agreement with the literature [43, 44], chloroquine (100 μM) and NH_4Cl (50 mM), used as control

tools, elevated the lysosomal pH in M_{LPS} to a comparable degree as 10 nM archazolid (**Fig. 3A**). A similar result was obtained, when changes of pH in organelles were monitored using the pH sensitive LysoTracker® probe and fluorescence microscopy (**Fig. 3B**). Note that the structurally distinct V-ATPase inhibitor apicularen A also elevated the pH in organelles of M_{LPS}, just like archazolid.

In order to investigate whether the increased release of TNFα by archazolid and other V-ATPase inhibitors was due to lysosomal pH alterations, we took advantage of chloroquine and NH_4Cl as tool compounds (that also elevated the lysosomal pH) and tested if these agents could increase TNFα release from M_{LPS} and M1 as well. Surprisingly, chloroquine (100 μM) and NH_4Cl (50 mM) failed to enhance TNFα release, but instead markedly repressed it in both M_{LPS} and M1 (**Fig. 3C**). Therefore, the data suggest that other mechanisms than solely elevation of lysosomal pH is responsible for the TNFα-upregulatory effects by archazolid and other V-ATPase inhibitors.

3.4. Archazolid affects TNFα release on the mRNA level and increases TNFα mRNA expression in classically-activated macrophages

The stimulatory effect of archazolid on TNFα release in classically-activated M1 was abrogated by co-incubation with dexamethasone (**Fig. 4A**), a glucocorticoid that interferes with TNFα gene expression. This finding led us to assume that archazolid may affect the expression of TNFα at the mRNA level. M(0) were pretreated with or without archazolid in the presence or absence of dexamethasone and 30 min later, LPS was added to obtain M_{LPS}. TNFα mRNA levels after 4 and 8 hrs were significantly higher in the presence of archazolid versus cells treated with LPS alone, and also after 1 and after 16, 20 and 24 hrs, archazolid led to elevated levels of TNF mRNA (**Fig. 4B**). Note that mRNA of IL-1β, studied as control, was not increased by archazolid (**Fig. 4B**). Coincubation with dexamethasone effectively repressed the up-regulatory effect of archazolid on TNFα mRNA expression (**Fig. 4B**). Experiments using actinomycin D that blocks mRNA

transcription showed that archazolid does not affect TNFα mRNA degradation as possible reason for elevation of mRNA levels (**Fig. 4C**).

3.5. Effects of archazolid on protein kinases and transcription factors involved in LPS-signaling and on ROS formation

Because archazolid seemingly enhances the LPS-induced expression of TNFα mRNA in M_{LPS}, it appeared possible that the compound modulates LPS-induced signal transduction pathways. We thus analyzed the phosphorylation of typical protein kinases (PK) that are integrated in LPS-induced TNFα expression. M(0) were pre-treated with archazolid or respective reference PK inhibitors, stimulated with LPS for 15 min and analyzed for the phosphorylated form of the kinases by Western blot. LPS caused phosphorylation of Akt, ERK-1/2, p38 MAPK, MEK-1/2, and MEK-3/6 (**Fig. 5A**) but archazolid (10 or 100 nM) failed to modulate their phosphorylation. The reference inhibitors LY294002 for Akt phosphorylation, U0126 for ERK, and SB203580 for p38 MAPK, used to validate the identity of respective phosphorylated kinases/proteins detected by Western blot, blocked LPS-induced phosphorylation, as expected (**Fig. 5A**). A tendency for stimulated SAPK/JNK phosphorylation (reference inhibitor SP600125) was observed for 10 and 100 nM archazolid, although statistical significance was not reached (**Fig. 5A**). However, archazolid (10 and 100 nM) clearly elevated the phosphorylation of the p65 subunit of the transcription factor NFκB in M_{LPS} without marked elevation of NFκB p65 expression (**Fig. 5B**). An NFκB-stimulatory effect of archazolid was also observed when the translocation of activated NFκB to the nucleus was monitored using immunofluorescence microscopy, in agreement with disappearance of the NFκB chaperon IκB (**Fig. 5C**). Note that the LPS-induced phosphorylation of other relevant transcription factors, that is, STAT-1 and STAT-3 in M1 was not elevated by archazolid (**Fig. 5D**).

Because ROS production is closely linked to NFκB and SAPK/JNK signaling [45], we investigated if archazolid could modulate LPS-induced ROS formation in macrophages. Pre-incubation of macrophages with 10 or 30 nM archazolid prior to LPS significantly increased ROS levels within 24 hrs whereas the NADPH oxidase inhibitor DPI diminished ROS formation induced by LPS, as expected, and also abrogated the elevated ROS levels caused by archazolid (**Fig. 6A**). Finally, we investigated if the elevated ROS and NFκB activation are involved in archazolid-induced TNFα release. In fact, DPI (5 μM) that reduced archazolid-elicited ROS formation as well as the NFκB activation inhibitor parthenolide (10 μM) efficiently prevented the stimulatory effects of archazolid plus LPS on TNFα release from macrophages (**Fig. 6B**).

3.6. Archazolid enhances M1-mediated cytotoxicity against MCF-7 cells in a microfluidically biochip assay

To investigate if treatment of macrophages by archazolid influences cancer cell viability in co-cultures, we utilized a microfluidically-supported biochip assay [32], designed as a dynamically perfused three-dimensional human tumour model (**Fig. 7A**). Macrophages, polarized towards M1 or M2 were added endothelial cells (ECs) and co-cultured in the upper chamber. The ECs are thought to separate the tumor from the blood cells mimicking the physiological barrier. MCF-7 cells, resembling the tumour component, were cultured underneath the EC-macrophage cell layer. EC-macrophage cell layers treated with archazolid (30 nM) for 30 min and subsequent incubation for 24 hrs significantly reduced the viability of MCF-7 cells in models containing M1, whereas in models containing M2 or in models devoid of macrophages, archazolid failed in this respect (**Fig. 7B, C**).

4. Discussion

Here we show that pharmacological targeting of V-ATPase causes selective elevation of TNFα production in classically-activated (i.e., M1 and M_{LPS}) human macrophage phenotypes. Intriguingly, the concomitant release of other cytokines (i.e. IL-1β, IL-6, IL-10) or chemokines (i.e. IL-8, MCP-1) in M1 and M_{LPS} was not elevated, and the corresponding cytokine/chemokine secretion of uncommitted M(0) and of alternatively activated M2 was not affected by archazolid. Because also other V-ATPase inhibitors such as bafilomycin and apicularen led to comparable effects, we suggest that interference with V-ATPase but not with an off-target of archazolid [46] is causative for the elevated TNFα levels. It appears that V-ATPase inhibition enhances the LPS- or LPS/INFγ-induced response of macrophages for TNFα expression, seemingly by augmenting LPS-evoked NFκB activation and ROS formation. Finally, in a biochip tumor model, archazolid caused reduced viability of MCF-7 breast cancer cells in co-culture with M1, but not with M2 or in absence of macrophages. Our data suggest that V-ATPase in classically-activated macrophages may play a regulatory role in TNFα expression implying a therapeutic potential for V-ATPase inhibitors to intervene with diseases where increased TNFα levels might be beneficial, such as cancer.

V-ATPase is ubiquitously expressed in mammals and was studied in macrophages before, where it was shown to play roles in lysosomal and cytoplasmic pH homeostasis [34, 42, 47, 48]. Also in our study, using archazolid for the first time as tool for investigating V-ATPase functions in macrophages, modulation of lysosomal pH homeostasis was apparent, since archazolid caused elevation of the pH in lysosomes. Of interest, in contrast to cancer cells that overexpress V-ATPase [5, 6] and where V-ATPase inhibition by archazolid induces cell death [18-20, 22, 49], the viability of human monocyte-derived macrophages was not impaired in response to V-ATPase interference by archazolid. Similarly, archazolid failed to reduce the viability of human primary monocytes [23].

Pharmacological manipulation of V-ATPase in macrophages by respective inhibitors such as bafilomycin A1 was reported before [13, 31, 35, 37, 38, 48, 50]. Many of these studies focused on osteoclasts [51], macrophage cell lines [31] and macrophages from rodents [37, 39, 48] and from other animals [52], but only few studies addressed V-ATPase functions in human primary macrophages [40, 50], and to the best of our knowledge there are no reports that studied the role of V-ATPase on cytokine/chemokine secretion of different macrophage phenotypes, such as M1 and M2. Therefore, one significant novelty of our study is the consideration of distinct human macrophage subtypes, that is, uncommitted M(0), classically-activated M1-like and so-called "resolving" M2-like macrophages that significantly differ in their bioactions [53]. In fact, we show here that blocking V-ATPase upregulates TNFα expression in classically-activated M1-like but not so in M(0) and M2 subtypes.

A previous study demonstrated that bafilomycin A1 reduced the amount of TNFα in LPS-activated alveolar macrophages from rabbits [11], and we recently found that archazolid suppresses TNFα secretion from human monocytes [23]. These opposing results versus our present data emphasize the need of distinguishing between monocyte/macrophage subsets. Recently, a differential role of V-ATPase in M1 and M2 was observed in phagosome pH regulation [40]. In line with our data are previous findings showing that V-ATPase suppresses NFκB and cytokine expression in LPS- or INFγ-activated macrophages [12]. It is noteworthy that among various cytokines and chemokines, solely TNFα was affected by archazolid. The reason for this effect is unknown but selective elevation of TNFα without concomitant increase of Il-1β via NFκB was also observed in LPS-stimulated murine peritoneal macrophages treated with the protein biosynthesis inhibitor cycloheximide [54]. Of interest, V-ATPase might be of importance for polarization towards M2 subsets. Thus, the lysosomal adaptor protein Lamtor1, which forms an amino-acid sensing complex with V-ATPase, was recently shown to be required for M2 polarization [55]. Moreover, V-ATPase was reported to modulate macrophage polarization in tumor-bearing mice [56], and inhibition of the V-ATPase a2

isoform in murine tumor cells delayed tumor growth by decreasing M2-like TAMs in the tumor microenvironment [57].

In our study, targeting of V-ATPase in classically-activated macrophages caused marked elevation of lysosomal pH, a typical effect of V-ATPase inhibitors that block the proton-pumping activity of V-ATPase [16, 41, 42]. Therefore, it appeared possible that lysosomal pH elevation is causative for increased TNFα expression. However, the control tool compounds chloroquine and NH_4Cl that are known to increase lysosomal pH in macrophages [43, 44], also elevated the pH in lysosomes of M_{LPS} comparable as archazolid, but TNFα release was not increased but rather impaired. These data suggest that simply elevation of lysosomal pH is probably not the cause for increased TNFα expression by targeting V-ATPase.

Our detailed analysis of PKs and transcription factors that are potentially involved in induction of TNFα expression [58] excluded the MEK1/2 – ERK, MEK3/6 – p38 MAPK, and Akt pathway as well as STAT-1 and -3 as signaling molecules since archazolid treatment of M_{LPS} did not increase phosphorylation/activation of these proteins. Instead, phosphorylation of the p65 subunit of NFκB and the nuclear translocation of NFκB p65 were markedly promoted by archazolid, along with almost complete disappearance of the NFκB chaperon IκB. Since NFκB regulates TNFα expression in macrophages at the transcriptional level [59, 60], we concluded that interference with V-ATPase by archazolid leads to elevation of TNFα mRNA expression. This is supported by the finding that the glucocorticoid dexamethasone which diminishes NFκB levels [59], completely prevented archazolid-induced TNFα mRNA levels. Note that besides NFκB also the SAPK/JNK was slightly activated by archazolid, a signaling molecule controlling TNFα translation in macrophages susceptible to dexamethasone [61]. Based on our experiments with actinomycin D the stability of TNFα mRNA was not affected by archazolid. Although TNFα is well known to augment NFκB signaling [62], it is unlikely that archazolid-induced TNFα is causative for increased NFκB activation, as the later was

elevated already 15 min upon exposure to archazolid while TNFα induction was delayed up to hours. Macrophages substantially produce ROS during bacterial killing to clear infections. In agreement with increased ROS formation in murine macrophages exposed to the V-ATPase inhibitors bafilomycin A1 or to concanamycin A [31], archazolid enhanced LPS-induced ROS formation in human macrophages, which was sensitive to the NDAPH oxidase inhibitor DPI. Of interest, DPI also blocked archazolid-induced TNFα release implying a requirement of ROS in this respect. In fact, ROS production was shown to be closely linked to NFκB and SAPK/JNK activity [45].

In summary, we showed that pharmacological targeting of V-ATPase has phenotype-specific consequences for human macrophage functions. Substantial secretion of TNFα is a hallmark of activated macrophages which is increased by V-ATPase inhibition. Although TNFα is a potent pro-inflammatory cytokine that contributes to excessive and unresolved inflammation, controlled temporal and spatial elevation of TNFα may be beneficial in cancer treatment. In fact, attempts have been made to stimulate macrophages for higher TNFα production, for example by TLR-9 activators and CpG oligodeoxynucleotides [63, 64] in order to promote immune reactions against the tumor. Our results from the biochip tumor model support anti-tumoral properties of archazolid in combination with M1 but not with M2. Together, V-ATPase inhibitors may have the appeal to act at least in two ways that could synergize in cancer therapy: (i) direct anti-tumoral effects against susceptible cancer cells and (ii) beneficial impact against tumors by phenotype-specific manipulation of macrophages in the tumor microenvironment.

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Conflict of Interest Statement: None declared.

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Figure legends

Figure 1. Expression and subcellular localization of V-ATPase in macrophages and effects of archazolid on macrophage viability and morphology. (A) Macrophages, obtained from human blood monocytes after differentiation with M-CSF (25 ng/mL) for six days, were stimulated with LPS (100 ng/mL), with LPS (100 ng/mL) plus IFNγ (100 ng/mL) or with IL-4 (20 ng/mL) for 24 hrs. Cells were permeabilized and incubated with an antibody against V-ATPase (green), followed by Alexa Fluor 488 goat anti-rabbit IgG. Nuclei were stained with DAPI (blue). (B) Macrophages were stimulated with LPS (100 ng/mL) plus IFNy (100 ng/mL) for 24 hrs, permeabilized and incubated with antibodies against v-ATPase (green) or LAMP-1 (red), followed by Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 555 goat anti-rabbit IgG; nuclei were stained with DAPI (blue); differential interference contrast. (C, D) Macrophages were pre-incubated with archazolid (1, 10, 100 nM (C) and 100 nM (D)), staurosporine (3 μM) or vehicle (0.1% DMSO) for 30 min and stimulated with LPS (100 ng/mL), with LPS (100 ng/mL) plus IFNγ (100 ng/mL) or with IL-4 (20 ng/mL) for 24 hrs. (C) Cell viability was determined by MTT assay. (D) Effects of archazolid (100 nM) on the morphology of macrophages. Microscopic images are shown as differential interference contrast. All results are representative of three independent experiments. Values are given as percentage of vehicle control (= 100%), means + SEM; n = 3. ***$P < 0.001$ vs. vehicle. ANOVA + Bonferroni post-hoc test.

Figure 2. Effects of V-ATPase inhibitors on cytokine and chemokine release from human macrophages. Human isolated blood monocytes were differentiated to macrophages with M-CSF (25 ng/mL) within six days. For analysis of cytokine release, macrophages were pre-incubated with archazolid, apicularen A, or bafilomycin A1 at the indicated concentrations, with dexamethasone (1 μM) or with vehicle (0.1% DMSO) for 30 min and then stimulated with LPS (100 ng/mL), LPS plus

IFN-γ, or IL-4 (20 ng/mL) for another 24 hrs. Cytokines and chemokines in the supernatants were analysed by ELISA. Values shown are percentages of vehicle control, means + SEM. *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle (DMSO). Paired t-test (A) or ANOVA + Tukey post-hoc test (B, C). (A) Archazolid was used at 100 nM. Absolute values of LPS + IFNγ-stimulated vehicle (100%) control: MCP-1: 98.3 ± 43.4 ng/mL, IL-1β: 1709 ± 1465 pg/mL, IL-6: 30.8 ± 9.3 ng/mL, IL-8: 52.2 ± 4.8 ng/mL, IL-10: 1211 ± 256 pg/mL, TNFα: 1992 ± 653 pg/mL. Absolute values of IL-4-stimulated vehicle (100%) control: MCP-1: 86.1 ± 34.6 ng/mL, IL-1β: 601 ± 403 pg/mL, IL-6: 11.3 ± 1.9 ng/mL, IL-8: 45.1 ± 4.8 ng/mL, IL-10: 819 ± 240 pg/mL, TNFα: 259 ± 87 pg/mL; n = 4. (B, C) Absolute value of LPS-stimulated vehicle (100%) control: 1383 ± 229 pg/mL (n = 6), absolute value of LPS + IFNγ-stimulated vehicle (100%) control: 1906 ± 273 pg/mL (B, n = 3) and 2347 ± 632 pg/mL (C, n = 6).

Figure 3. Effects of V-ATPase inhibition on the lysosomal pH of human macrophages and correlation with cytokine release. Human isolated blood monocytes were differentiated to macrophages with M-CSF (25 ng/mL) within six days. (A) Macrophages were incubated with FITC-dextran (0.5 mg/mL) for 60 min, preincubated with archazolid at the indicated concentrations, ammonium chloride (50 mM), chloroquine (100 µM), or vehicle (0.1% DMSO) for 30 min and stimulated with LPS (100 ng/mL) for 24 hrs. FITC-Dextran uptake into lysosomes was determined by measurement of emission at 520 nm or by analysis of immunofluorescence pictures (FITC-dextran: green; differential interference contrast). (B) Macrophages were pre-incubated with archazolid (100 nM), apicularen A (100 nM), ammonium chloride (50 mM) or vehicle (DMSO) for 30 min and stimulated with LPS (100 ng/mL) for 24 hrs. Macrophages were incubated with LysoTracker (50 nM; red) for 60 min and staining of acidic organelles was observed using a fluorescence microscope. (C) Macrophages were pre-incubated with archazolid (10 nM), ammonium chloride (50 mM), chloroquine (100 µM) or vehicle (DMSO) for 30 min and stimulated with LPS

(100 ng/mL) or LPS (100 ng/mL) plus IFNγ (100 ng/mL) for 24 hrs. TNFα levels in supernatants were analysed by ELISA. Values are given as percentage of vehicle control (= 100%), means + SEM; n = 8 (A, left panel), n = 3 (C). *P < 0.05, **P < 0.01, ***P < 0.001 vs. the LPS-stimulated vehicle (= 100%). ANOVA + Bonferroni post-hoc test. Pictures shown are representative of three independent experiments.

Figure 4. Effects of archazolid on TNFα mRNA expression and degradation. (A) Macrophages, obtained from human blood monocytes after differentiation with M-CSF (25 ng/mL) for six days, were pre-incubated with archazolid (100 nM), with or without dexamethasone (1 μM) or with vehicle (0.1% DMSO) for 1 hr and then stimulated with LPS (100 ng/mL) for 8 hrs. Levels of TNFα protein in supernatants were analysed by ELISA. Values are given as percentage of vehicle control (100%, DMSO), means + SEM; n = 3. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the LPS-stimulated vehicle control. ANOVA + Bonferroni post-hoc test. (B) Macrophages were pre-incubated with archazolid (100 nM), with archazolid (100 nM) plus dexamethasone (1 μM) or with vehicle (0.1% DMSO) for 1 hr and then stimulated with LPS (100 ng/mL). At the indicated time points mRNA was extracted and determined by RT-qPCR. mRNA levels of TNFα were normalized against those of B2M. Values shown are given as fold increase over control, means + SEM; n = 4. **P < 0.01, ***P < 0.001 vs. the unstimulated archazolid-treated sample at time point = 0 hrs. ANOVA + Tukey post-hoc test. (C) Macrophages were stimulated with LPS (100 ng/mL) for 24 hrs and incubated with archazolid (10 nM) or vehicle (0.1% DMSO) for 60 min. Transcription was stopped by actinomycin D (5 μg/mL) and macrophages were further incubated for the indicated times. TNFα mRNA levels were determined by RT-qPCR. Values are given as percentage of control (100%), means + SEM; n = 4.

Figure 5. Effects of archazolid on activation of protein kinases and transcription factors involved in LPS signalling pathways. (A) Macrophages, obtained from human blood monocytes after differentiation with M-CSF (25 ng/mL) for six days, were first starved for 6 hrs and then pre-incubated with archazolid at the indicated concentrations, LY294002 (10 μM), U0126 (3 μM), SB203580 (10 μM), SP600125 (10 μM), or vehicle (0.1% DMSO) for 30 min, and stimulated with LPS (100 ng/mL) for 15 min. Protein phosphorylation or expression in cell lysates was analysed by Western blotting; GAPDH or the respective unphosphorylated proteins were used for normalization. Representative Western blots of 4 independent experiments are shown; data (densitometric analysis) are means + SEM; n = 4. (B, D) Macrophages were pre-incubated with archazolid at the indicated concentrations, parthenolide (10 μM) or vehicle (0.1% DMSO) for 30 min and stimulated with LPS (100 ng/mL) for 15 min. (B) NFκB p65 phosphorylation (left panel) and expression (right panel) or (D) STAT-1 and -3 phosphorylation in cell lysates was analysed by Western blotting; β-actin was used for normalization. Representative Western blots of 4 independent experiments are shown; data (densitometric analysis) are means + SEM; n = 4 (C) Macrophages were seeded onto coverslips, pre-incubated with archazolid (10 nM), or vehicle (0.1% DMSO) for 30 min and stimulated with LPS (100 ng/mL) for 15 min. Fixed and permeabilized cells were stained with antibodies against IκB (Alexa Fluor 555, red) and NFκB p65 (Alexa Fluor 488, green), nuclei were stained with DAPI (blue). Representative immunofluorescence images of three independent experiments are shown.

Figure 6. Archazolid elevates ROS formation in LPS-stimulated macrophages; effects of ROS and NFκB inhibitors on archazolid-induced TNFα release. (A) Macrophages, obtained from human blood monocytes after differentiation with M-CSF (25 ng/mL) for six days, were pre-incubated with archazolid at the indicated concentrations, DPI (5 μM), or with vehicle (0.1% DMSO) for 30 min and stimulated with LPS (100 ng/mL) for 24 hrs. Fluorescence of the oxidised 2′,7′-dichlorofluorescin diacetate (5 μg/mL) was measured at an excitation wavelength of 485 nm and an

emission wavelength of 535 nm. Values shown are percentages of vehicle control, means + SEM; n = 4. *P < 0.05, ***P < 0.001 vs. the LPS-stimulated vehicle control (100%, DMSO); $^{\#\#}$P < 0.01 vs. cells treated with LPS plus 10 nM archazolid. (B) Macrophages were pre-incubated with DPI (5 μM), parthenolide (10 μM, parth) or vehicle (0.1% DMSO) for 15 min. Then, cells were treated with vehicle or archazolid (30 nM), and after another 15 min, cells were treated with LPS (100 ng/mL) for 24 hrs. TNFα in the supernatants was analysed by ELISA. Values shown are percentages of LPS-stimulated controls (= 100%), means + SEM. ***P < 0.001 vs. LPS plus vehicle (DMSO); $^{\#\#\#}$P < 0.001 vs. cells treated with LPS plus archazolid. ANOVA + Tukey post-hoc test.

Figure 7. Archazolid-treatment of M1 reduces the viability of MCF-7 cells in in a biochip-based tumor model. (A) Schematic view of the tumor biochip model. HUVEC and polarized macrophages were placed on a membrane in the upper chamber of a microfluidically-supported biochip, while MCF-7 cells are cultured underneath the vascular layer of the biochip. (B, C) Viability of MCF-7 cells by Calcein-AM or propidium iodide staining after 24 h incubation with M1 (macrophages + LPS + INFγ), M2 (macrophages + IL-4) or in absence of macrophages that had been pretreated with 30 nM archazolid or vehicle (0.1% DMSO) for 30 min. (B) Representative images of three independent experiments; green: Calcein-AM staining of viable MCF-7 cells; red: propidium iodide staining of dead MCF-7 cells. (C) Mean fluorescence intensity (MFI) of the Calcein-AM staining of MCF-7 cells was measured by random field analysis of 40 regions of interest per experiment. Left panel, presence of M1 (macrophages + LPS + INFγ) or M2 (macrophages + IL-4), as indicated. Right panel, absence of macrophages. Data are means + SEM, n=3; *p<0.05. n.s., not significant.