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Targeting V-ATPase in primary human monocytes by archazolid potently represses the classical secretion of cytokines due to accumulation at the endoplasmic reticulum

Olga Scherer^a, Heinrich Steinmetz^b, Christoph Kaether^c, Christina Weinigel^d, Dagmar Barz^d, Hartmut Kleinert^e, Dirk Menche^f, Rolf Müller^g, Carlo Pergola^a, Oliver Werz^{a,*}

^aChair of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich Schiller University Jena, Philosophenweg 14, 07743 Jena, Germany; E-mail addresses: olga.scherer@uni-jena.de; pergolacarlo@gmail.com; oliver.werz@uni-jena.de

^bHelmholtz Centre for Infection Research, Braunschweig, Germany; E-Mail address: info@helmholtz-hzi.de

^cLeibniz Institute for Age Research - Fritz Lipmann Institute, Jena, Germany; E-Mail address: ckaether@fli-leibniz.de

^dInstitute of Transfusion Medicine, University Hospital Jena, Jena, Germany; E-mail addresses: christina.weinigel@med.uni-jena.de; dagmar.barz@med.uni-jena.de

^eJohannes Gutenberg University, Mainz, Germany; E-Mail address: kleinert@uni-mainz.de

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

^gSaarland University, Saarbrücken, Germany; 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

The macrolide archazolid inhibits vacuolar-type H(+)-ATPase (V-ATPase), a proton-translocating enzyme involved in protein transport and pH regulation of cell organelles, and potently suppresses cancer cell growth at low nanomolar concentrations. In view of the growing link between inflammation and cancer, we investigated whether inhibition of V-ATPase by archazolid may affect primary human monocytes that can promote cancer by sustaining inflammation through the release of tumor-promoting cytokines. Human primary monocytes express V-ATPase, and archazolid (10 to 100 nM) increases the vesicular pH in these cells. Archazolid (10 nM) markedly reduced the release of pro-inflammatory (TNF- α , interleukin-6 and -8) but also of anti-inflammatory (interleukin-10) cytokines in monocytes stimulated with LPS, without affecting cell viability up to 1000 nM. Of interest, secretion of interleukin-1 β was increased by archazolid. Comparable effects were obtained by the V-ATPase inhibitors bafilomycin and apicularen. The phosphorylation of p38 MAPK and ERK-1/2, Akt, SAPK/JNK or of the inhibitor of NF κ B (I κ B α) as well as mRNA expression of IL-8 were not altered by archazolid in LPS-stimulated monocytes. Instead, archazolid caused endoplasmic reticulum (ER) stress response visualized by increased BiP expression and accumulation of IL-8 (and TNF- α) at the ER, indicating a perturbation of protein secretion. In conclusion, by interference with V-ATPase, archazolid significantly affects the secretion of cytokines due to accumulation at the ER which might be of relevance when using these agents for cancer therapy.

Key words: vacuolar-type H(+)-ATPase, archazolid, cytokines, monocytes, interleukin, inflammation.

Abbreviations used: BFA, brefeldin A; CHX, cycloheximide; DECP, diethylpyrocarbonate; ER, endoplasmic reticulum; IL, interleukin; PBMC, peripheral blood mononuclear cells; V-ATPase, vacuolar-type H(+)-ATPase.

1. Introduction

Monocytes are a major component of innate immunity that traffic via the bloodstream to peripheral tissues and upon recruitment protect the host against pathogenic microorganisms [1]. However, recruited monocytes also contribute to the pathogenesis of inflammatory and degenerative diseases, including cancer. For example, monocytes can infiltrate the tumor microenvironment and support the persistence of an inflammatory milieu (e.g., by production of cytokines) and thus, contribute to all phases of the cancer progression [2, 3]. In particular, the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6 and IL-8 produced by activated and infiltrating monocytes are key players of inflammatory disorders and also promote tumor development [4, 5]. Thus, anti-inflammatory drugs represent valid complementary strategies also for cancer prevention and therapy [2]. Recent studies suggest that the combination of direct cytotoxic activity on tumor cells with a capacity to favorably modify the inflammatory tumor microenvironment is an appealing option for treatment of diverse cancers with clinical relevance [6], as shown for the natural compound trabectedin [7]. However, the identification of novel and relevant targets is currently required in order to simultaneously hit viability of neoplastic cells and inflammation in immune 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 [8]. V-ATPases participate in physiological processes such as receptor-mediated endocytosis, intracellular trafficking, protein degradation and processing, but they are also implicated in several diseases including osteoporosis, renal tubular acidosis, and malignant neoplasms [9]. In cancer, 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, the acquisition of metastatic cell phenotypes and tumor invasiveness [10]. Accordingly, specific inhibitors of V-ATPases decreased the acidity of tumors, reduced

survival of tumor cells and tumor metastasis, and impaired chemoresistance [11, 12]. Therefore, V-ATPase is considered as promising target in various types of cancer, but whether it may simultaneously regulate relevant inflammatory pathways in primary human monocytes is unknown.

Here, we made use of the natural V-ATPase inhibitor archazolid to analyze whether and how the release of pro-inflammatory cytokines related to tumor-promoting inflammation is regulated by V-ATPase in primary monocytes from healthy humans. In fact, archazolid binds to V-ATPase [13, 14] and recent work has shown that archazolid induced apoptosis and reduced migration of invasive tumor cells, decreased metastatic dissemination of breast tumors and spatially restricted activation of EGFR and Rho-GTPase Rac1 *in vivo* [15]. In various highly invasive tumor cell lines, archazolid led to apoptosis due to activation of the hypoxia-inducible factor-1 α (HIF1 α), autophagy [16], and anoikis induction [17]. Thus, archazolid currently represents a promising candidate for direct targeting of tumor cells via V-ATPase inhibition, but the analysis of its effects on inflammation is required for a comprehensive understanding of the therapeutic potential of V-ATPase inhibition.

2. Materials and Methods

2.1. Materials

Archazolid B was isolated from *Archangium gephyra* as described [18]. Apicularen A was obtained from the natural compound library of HZI/HIPS (Braunschweig, Germany). RPMI 1640 medium with L-glutamine, penicillin, streptomycin, and human serum were from PAA Laboratories (Pasching, Germany). For in-house made ELISA, the capture and detection antibodies were from R&D Systems (Abington, UK). U0126 and SP600125 were from Enzo Life Sciences (Lörrach, Germany) and staurosporine from Calbiochem (Merck, Darmstadt, Germany). Bafilomycin, dexamethasone, cycloheximide (CHX) and all other fine chemicals were from Sigma-Aldrich (Steinheim, Germany), unless indicated otherwise.

2.2. Isolation of blood cells and Mono Mac 6 cell culture

Leukocyte concentrates, prepared from freshly withdrawn peripheral blood of healthy adult human donors who had not taken any anti-inflammatory drugs for the last 10 days, with consent, were obtained from the Institute of Transfusion Medicine at University Hospital Jena, Germany. Platelets and peripheral blood mononuclear cells (PBMC) were isolated by dextran sedimentation and centrifugation on LSM 1077 lymphocyte separation medium (PAA Laboratories, Pasching, Germany), as described [19, 20]. To obtain monocytes, PBMC were collected, washed two times with cold PBS, and monocytes were separated by adherence for 1.5 h at 37 °C to culture flasks (2×10^7 cells/mL RPMI 1640 medium containing 2 mM L-glutamine and 100 U/mL penicillin plus 100 µg/mL streptomycin). Monocytes were finally resuspended in PBS, with a purity of >85%, defined by forward- and side-light scatter properties and detection of the CD14 surface molecule by flow cytometry (BD FACS Calibur, Heidelberg, Germany).

The human monocytic MM6 cell line (kindly provided by Dr. Thorsten Maier, University of Frankfurt, Germany) was cultured in RPMI 1640 medium, supplemented with heat-inactivated fetal calf serum (FCS, 10%, v/v), 100 U/mL penicillin, 100 µg/mL streptomycin, 10 µg/mL insulin, 1 mM oxaloacetic acid, 1 mM sodium pyruvate and 1×non-essential amino acids at 37 °C and 5% CO₂.

2.2. Determination of cell viability

Cell viability was assessed by MTT assay as described [21]. Briefly, monocytes were preincubated (30 min, 37 °C, 5% CO₂) with compounds and stimulated with 10 ng/mL LPS or left untreated for the indicated times. MTT was added, cells were further incubated for 2 h, and lysed in a buffer containing 10% (w/v) SDS. Staurosporine, an inducer of apoptosis, was used as positive control.

2.3. Determination of phosphorylation of p38 MAPK, ERK-1/2, Akt, SAPK/JNK, and IκB; BiP expression; Western blot analysis

Monocytes were incubated in RPMI 1640 medium plus 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine without serum overnight for starvation. Cells were pre-incubated with compounds or DMSO (30 min, 37 °C), 2% human serum was added and cells were stimulated with LPS (100 ng/mL) for 5 min (p38 MAPK, ERK-1/2, Akt, SAPK/JNK) or 15 min (IκB). For analysis of BiP expression, monocytes were plated in monocyte medium, pre-incubated with compounds or DMSO (30 min, 37 °C) and stimulated with LPS (1 µg/ml) for 24 h. Cells were placed on ice, washed twice with PBS and lysed by NP-40 (1%) as described [19]. 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, 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) bromphenol blue), the samples were boiled for 5 min at 96 °C. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK). After blocking with 5% (w/v) BSA or low fat powdered milk (Roth, Karlsruhe, Germany), membranes were incubated with primary antibodies overnight at 4 °C. Antibodies against I κ B α (1:800), phospho-I κ B α (1:1000), phospho-p38 (1:1000), ERK-1/2 (1:1000), phospho-ERK-1/2 (1:2000), phospho-Akt (1:1000), phospho-SAPK/JNK (1:1000), BiP (1:1000) (Cell Signaling Technology, MA, USA), β -actin (1:1000, Santa Cruz Biotechnology, Heidelberg, Germany) were used. Membranes were washed and incubated with fluorescently-labeled secondary antibodies (LI-COR Biosciences, Bad Homburg, Germany) for 1 h at RT in the dark. Proteins were detected using the Odyssey imaging system (LI-COR Biosciences, Bad Homburg, Germany).

2.4. Subcellular fractionation

For cell fractionation experiments, 3×10^7 monocytes or 1×10^7 MM6 cells were resuspended in 200 μ l ice-cold TKM buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 25 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 60 μ g/mL soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride and 10 μ g/mL leupeptin), and sonicated (10 strokes, lowest intensity) on ice. Cell debris was spun down (600 $\times$ g, 10 min, 4 °C), and supernatants were mixed with 4 $\times$ SDS loading buffer and boiled for 5 min. To separate ER- and Golgi-containing cellular fractions, the supernatants after 600 $\times$ g centrifugation were spun at 13 000 $\times$ g (10 min, 4 °C). The pellet (P13) containing the ER [22], was again resuspended in 200 μ l TKM buffer, sonicated on ice and boiled after 4 $\times$ SDS loading buffer addition. The supernatant (S13) was centrifuged at 100 000 $\times$ g (1.5 h, 4 °C). The resulting pellet (P100, containing Golgi) was also resuspended in 200 μ l TKM buffer and sonicated on ice. Both, P100 and the supernatant (S100) were mixed with 4 $\times$ SDS loading buffer and boiled for 5 min.

SDS-PAGE and Western-Blot were performed as described above. Antibodies against the c-subunit of V-ATPase (1:1000 anti-ductin, Biozol, Eching, Germany), Calnexin (1:1000, Merck, Darmstadt, Germany), GM130 (1:1000, BD Biosciences, Heidelberg, Germany), LAMP1 (1:1000, Abcam, Cambridge, UK), β -actin and β -tubulin (1:1000, Cell Signaling Technology, MA, USA) were used. Bands were visualized and band intensities were quantified (after background correction) using the Odyssey imaging system (LI-COR Biosciences, Bad Homburg, Germany).

2.5. Determination of intracellular and extracellular cytokine levels

Monocytes ($1.5 \times 10^6/\text{mL}$) were resuspended in monocyte medium (RPMI 1640 containing 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 2 mM L-glutamine and 2% (v/v) human serum). After 1.5 h at 37 °C and 5% CO₂, test compounds or vehicle were added and 30 min later, cells were stimulated with 10 ng/mL LPS for 4 h (IL-8, TNF- α) or 18 h (IL-6, IL-1 β , IL-10). For measurement of extracellular cytokine levels supernatants were collected after centrifugation (2000 $\times g$, 4 °C, 10 min). For analysis of intracellular cytokine levels cells were centrifuged (315 $\times g$, 4 °C, 10 min), lysed using 1% (v/v) Triton X-100 buffer and lysates were centrifuged (14,000 $\times g$, 1 min, 4 °C). Cytokines were either analyzed by ELISA using commercially available kits (IL-1 β : Raybiotech, Norcross, GA; IL-8, TNF- α , IL-6: Assaypro, St. Charles, MO) according to manufacturer's specifications, or by in-house made ELISA (IL-10, IL-1 β , IL-8, TNF- α , IL-6).

2.6. Immunofluorescence microscopy

PBMC ($1 \times 10^7/\text{ml}$) were seeded on glass coverslips and incubated for 1.5 h at 37 °C at 5% CO₂. After monocyte attachment, residual cells were removed by washing with PBS. Monocytes were pre-incubated with test compounds (30 min) and then stimulated with LPS (10 ng/mL) for 4 h at 37 °C and 5% CO₂. Cells were fixed with 4% formaldehyde solution in PBS, free formaldehyde was

quenched with 50 mM ammonium chloride for 10 min and permeabilized with 0.2% (v/v) Triton X-100 (Roth, Karlsruhe, Germany). After blocking with 10% (v/v) non-immune goat serum (Invitrogen, CA), samples were first incubated with rabbit anti-IL-8 (1:200, Santa Cruz Biotechnology, Heidelberg, Germany) antibody overnight at 4 °C, and then with antibodies either against the *trans*-Golgi apparatus (1:200, anti-TGN38, Santa Cruz Biotechnology), *cis*- and *medial*-Golgi apparatus (1:500, anti-Giantin, Enzo Life Sciences, Lörrach, Germany), the ER-Golgi intermediate compartment (1:500, anti-ERGIC-53, Enzo Life Sciences, Lörrach, Germany), the ER (1:200, anti-Calnexin, Merck, Darmstadt, Germany) or the ER-exit sites (1:200, anti-Sec31A, BD Biosciences, Heidelberg, Germany) for 1 h at RT. The samples were then stained with goat anti-rabbit IgG Alexa Fluor 488 (1:1500) and anti-mouse IgG Alexa Fluor 555 (1:1000) antibodies (Invitrogen, CA) for 10 min at RT. DNA was stained using DAPI (0.7 µg/mL) for 3 min at RT. Samples were placed on microscope slides using Mowiol and analyzed by an Axio Observer Z1 inverted microscope (Carl Zeiss, Jena, Germany). Samples without primary antibodies were prepared as controls. For visualization of fluorescein isothiocyanate (FITC)-dextran uptake in monocytes, cells were seeded on coverslips, loaded with FITC-dextran and incubated with archazolid as described in the FITC-dextran method. After formaldehyde fixation of the cells, nuclei were stained using DAPI and the coverslips were mounted on glass slides (Roth, Karlsruhe, Germany) with Mowiol containing 0.25% n-propyl gallate (Sigma-Aldrich). The fluorescence was visualized with a Zeiss Axio Observer.Z1 microscope and a LCI Plan-Neofluar 63x/1.3 Imm Corr DIC M27 objective or a Plan-Apochromat 100x/1.40 Oil 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 to TIF by the AxioVision 4.8 software.

2.7. Relative quantification of IL-8 RNA expression in monocytes

Monocytes ($1.5 \times 10^6/\text{mL}$) were preincubated with archazolid (10 nM) or DMSO for 30 min and stimulated with 10 ng/mL LPS for indicated times or left untreated at 37 °C and 5% CO₂. Total RNA from monocytes was isolated by guanidinium thiocyanate/phenol/chloroform extraction as described [23]. cDNA was generated by reverse transcription. The cDNA was amplified by PCR and quantified using the MyiQ Singl-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Munich, Germany). To calculate the relative IL-8 mRNA expression (normalized to GAPDH) the $2^{(\Delta\Delta C(T))}$ method was used (23). Primers used: human GAPDH forward primer 5'-CCCATGTTTCGTCATGGGTGT-3', human GAPDH reverse primer 5'-TGGTCATGAGTCCTTCCACGATA-3', human IL-8 forward primer 5'-GCCGTGGCTCTCTTGGC-3', human IL-8 reverse primer 5'-TAGCACTCCTTGGCAAACTGC-3' (Eurofins MWG Operon, Ebersberg, Germany). TaqMan probes: human GAPDH probe 5'-CTGCACCACCAACTGCTTAGCACCC-3', human IL-8 probe 5'-CTTCCTGATTTCTGCAGCTCTGTGTGAAGG-3', 5'FAM/3'TAMRA (Biomers, Ulm, Germany).

2.8. Analysis of vesicular & cytosolic pH in monocytes

The pH measurements were performed as reported [24] and compositions of sodium and potassium buffers were used as described [25]. For measurements of cytosolic pH, monocytes ($1.25 \times 10^7/\text{mL}$) were incubated with compounds for 4 h at 37 °C and 5% CO₂. During the last 30 min of incubation the pH-sensitive dye BCECF-AM (0.1 μM, Life Technologies, OR) in sodium buffer was added. Cells were then washed with PBS and sodium buffer was added. The fluorescence was excited at 450 nm (not pH-sensitive, isosbestic point) and 485 nm (pH-sensitive) and the emission at 520 nm was recorded using a fluorescence microplate reader (Novostar, BMG Labtechnologies, Offenburg, Germany). The ratio of fluorescence intensities was calculated. *In situ* fluorescence calibration with

cells was performed using the K^+/H^+ ionophore nigericin (20 μ M, Enzo Life Sciences, Lörrach, Germany) in the presence of potassium buffer containing depolarizing K^+ concentrations and pH values ranging from 6.5 to 7.

For measurements of vesicular pH, monocytes were incubated with 0.5 mg/mL FITC-dextran (70 000 MW) for 1 h. Cells were washed with PBS and incubated with test compounds for 4 h. 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 ionophores nigericin (10 μ M) and monensin (20 μ M) in potassium buffer at pH values between 4.5 and 7.5.

For analysis by Lysotracker staining, monocytes (2.5×10^5 /mL) were plated into glass bottom dishes (MatTek Corporation, MA). After adherence, cells were treated with test compound or vehicle for 4 h. After washing and resuspension in PBS (plus 1 mg/ml glucose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$), monocytes were stained with a fluorescent Lysotracker probe (50 nM, Life Technologies, OR) for 5 min and red fluorescence of the accumulated probe in acidic cell organelles was imaged using the Axio Observer Z1 microscope.

2.9. Statistics

The data are presented as mean $\pm$ SEM of n experiments. Statistical analysis was performed by one-way ANOVA for independent or correlated samples followed by Bonferroni post hoc test or by Student t test [26]. Data were analyzed with GraphPad InStat program (GraphPad Software, CA). A P value of < 0.05 was considered significant.

3. Results

3.1. Monocytes express V-ATPase and respond to V-ATPase inhibition by archazolid without loss of cell viability

In order to ensure that primary human monocytes can respond to archazolid via V-ATPase, we first analyzed the occurrence of the V-ATPase protein in these cells. V-ATPase protein (c subunit) was detected in total cell lysates of primary PBMC and monocytes from human peripheral blood, whereas in platelets for instance, V-ATPase was essentially absent (**Fig. 1A**). However, the amount of V-ATPase in primary monocytes was lower versus transformed monocytic MM6 cells. After high-speed centrifugation ($100\,000\times g$) of the monocyte lysates, V-ATPase protein was essentially in the pelletable fraction (**Fig. 1A**). After differential centrifugation at $13\,000\times g$ and $100\,000\times g$, V-ATPase was found in the pellet of the $13\,000\times g$ (P13) but not in the pellet (P100) after $100\,000\times g$ centrifugation, thus co-sedimenting with markers of ER and lysosomal vesicles (i.e., calnexin and LAMP1, respectively), but not with the Golgi marker GM130; no significant amounts of V-ATPase protein was detected in the supernatants (S100) (**Fig. 1C**).

Next, we investigated whether archazolid functionally interferes with V-ATPase in monocytes and thus, elevates the vesicular pH by blocking V-ATPase-dependent proton transport into vesicles. Two methods were applied: (I) fluorescence microscopy using the LysoTracker probe which stains acidic compartments, and (II) fluorescence measurement of internalized FITC-dextran, which is pH-dependent. The results show that within 4 h, 10 nM archazolid causes a slight, and at 100 nM archazolid a significant increase of the pH of otherwise acidic cell organelles (**Fig. 2A, B**). These effects occurred in a concentration-dependent manner and were observed also for the structurally unrelated V-ATPase inhibitor apicularen (10 and 100 nM) (**Fig. 2B**). Note that the cytosolic pH of

archazolid-treated monocytes (10 or 100 nM archazolid) was instead not increased but slightly decreased, as expected (**Fig. 2B**).

As an increase of the vesicular pH by V-ATPase inhibitors is proposed to cause cancer cell death [10], we analyzed monocyte viability after archazolid treatment using the MTT assay. Of interest, archazolid up to 1000 nM failed to induce significant monocyte death within 24 h, whereas staurosporine (3 μ M, used as positive control) caused almost complete loss of cell viability (**Fig. 2C**). Also, the V-ATPase inhibitor apicularen (10-1000 nM) was not cytotoxic for monocytes (**Fig. 2C**). Together, primary monocytes express functional V-ATPase, and inhibitors of V-ATPase increase the vesicular pH, but in contrast to cancers cells, V-ATPase inhibitors cause no loss of viability.

3.2. Archazolid modulates cytokine release in activated monocytes without affecting related protein kinase signaling

We next investigated if archazolid alters the release of inflammation-related cytokines in monocytes. In agreement with the literature [27], LPS caused release of the pro-inflammatory TNF- α , IL-1 β , IL-6 and IL-8 and the anti-inflammatory IL-10 from primary monocytes (**Fig. 3**). Pretreatment with archazolid impaired LPS-induced release of TNF- α , IL-6, and IL-10 with effective concentrations in the range of 1 – 10 nM (reaching a plateau at higher concentrations) and similar efficiency as the anti-inflammatory compound dexamethasone (1 μ M) (**Fig. 3A**). Of interest, archazolid markedly suppressed IL-8 release, which was instead not affected by dexamethasone, and similar effects on IL-8 were observed for the V-ATPase inhibitors apicularen and bafilomycin (**Fig. 3A,B**). The protein synthesis inhibitor cycloheximide (CHX, 5 μ M) caused a comparable effect as the V-ATPase inhibitors. Intriguingly, archazolid promoted the LPS-induced release of IL-1 β , which was significant at 10 and 100 nM, whereas dexamethasone suppressed IL-1 β liberation

(**Fig. 3C**). Also bafilomycin and apicularen showed a tendency to increase LPS-induced IL-1 β release (**Fig. 3C**).

We next investigated the phosphorylation of signaling molecules involved in LPS-induced cytokine expression, such as p38 MAPK, Akt, ERK-1/2, SAPK/JNK, and I κ B α [27], which might be repressed by archazolid concomitantly with impaired cytokine release. However, the LPS-induced phosphorylation of these proteins was not diminished by archazolid (**Fig. 3D**), suggesting that LPS signaling down to the recruitment of relevant transcription factors (e.g., NF- κ B) is not targeted by V-ATPase inhibition.

3.3. Inhibition of V-ATPase by archazolid causes intracellular accumulation of IL-8 in activated monocytes

We aimed to mechanistically analyze the effect of archazolid on pro-inflammatory cytokines and since most potent inhibition was observed for IL-8 release, IL-8 (and for further confirmation also TNF- α) was used as model cytokine for subsequent analysis. To exclude interference of archazolid with cytokine de-novo biosynthesis at the transcriptional level we analyzed the expression of IL-8 mRNA in LPS-stimulated monocytes. LPS induced a robust increase of IL-8 mRNA in monocytes with maximum at 4 h and treatment of the cells with 10 nM archazolid did not impair this response (**Fig. 4A**), implying that V-ATPase inhibition does not repress IL-8 transcription. Next, we investigated if archazolid causes repression of IL-8 protein secretion. Thus, monocytes were isolated 4 h after treatment with LPS in the presence or absence of archazolid, and IL-8 protein released into the medium as well as the IL-8 content in the cells was assessed. Substantial amounts of IL-8 were found in the incubation medium of LPS-treated monocytes, and only low amounts of the cytokine remained in the cell (**Fig. 4B**). However, co-incubation with archazolid (1 to 10 nM) reduced the amount of secreted IL-8 but concomitantly increased the intracellular portion. A similar

effect was observed when TNF- α was analyzed (not shown). The same pattern was observed for apicularen and bafilomycin (10 nM, each), whereas CHX impaired in contrast both extra- and intracellular IL-8 levels, as expected (**Fig. 4B**).

V-ATPase inhibition by archazolid may block secretion of de-novo synthesized IL-8, presumably by increasing the pH in the secretory pathway. We thus investigated the extra- and intracellular IL-8 distribution pattern of LPS-stimulated monocytes in the presence of suitable tool compounds. Brefeldin A (BFA) that inhibits secretion by fusing the Golgi and the ER [28] also led to accumulation of intracellular IL-8 and impaired extracellular release, as expected (**Fig. 4C**). Similarly, the Na⁺/H⁺ antiporter monensin that blocks intracellular protein transport [29], led to an IL-8 distribution pattern as found for archazolid, that is, elevated IL-8 protein in the cell but reduced levels in the extracellular space (**Fig. 4C**).

Next, we confirmed archazolid-mediated intracellular IL-8 accumulation in monocytes by IF microscopy. In unstimulated and LPS-treated monocytes, IL-8 (green staining) localized to a compact structure, presumably the Golgi-area, partially “encircled” by the kidney bean-shaped nucleus (DAPI-stained, blue). Such a staining is typical for a secreted protein and was also observed for TNF- α (not shown). This IL-8 distribution pattern was essentially not altered for cells treated with CHX (**Fig. 4D**). However, archazolid caused a massive enrichment of IL-8 (and of TNF- α , not shown) within the entire non-nuclear environment, in addition to the juxtannuclear, compact structure. A similar pattern was observed for monensin-treated monocytes, whereas BFA caused rather homogenous distribution of IL-8 without marked accumulation in the Golgi-area, consistent with the disassembly of the Golgi caused by BFA (**Fig. 4D**).

3.4. Correlation between increased vesicular pH and intracellular accumulation of IL-8

We speculated that the intracellular IL-8 accumulation is caused by an increase of the vesicular pH after archazolid treatment. Therefore, we compared the effects of archazolid on vesicular pH and IL-8 secretion with those of NH₄Cl and chloroquine that are known to elevate the pH of cell organelles. NH₄Cl seemingly increased the vesicular pH (**Fig. 5A**) in LPS-stimulated monocytes, and in parallel retained LPS-induced IL-8 protein in the cell at the expense of released IL-8 (**Fig. 5B**), a pattern observed also for archazolid. Also 100 μ M chloroquine also slightly elevated the vesicular pH, but in contrast to archazolid and NH₄Cl, only the extracellular IL-8 was diminished without concomitant IL-8 accumulation in the cell.

3.5. Archazolid B causes accumulation of IL-8 at the ER and induces ER stress response

Using selective marker proteins of subcellular compartments/organelles related to classical protein secretion from ER via the Golgi into the extracellular space (**Fig. 6A**), we tested where IL-8 accumulates upon archazolid treatment. For visualization of the Golgi-area we used TGN38 and giantin, marking the *trans*-Golgi network or the *cis*- and *medial*-Golgi, respectively. Both stained the compact structure encircled by the bean-shaped nucleus and colocalized with IL-8, confirming that IL-8 is enriched in the Golgi-area (**Fig. 6B**). Both markers did not co-localize with the archazolid-induced IL-8 accumulation throughout the cell, nor did ERGIC-53, a marker of the ER-Golgi intermediate compartment. Also visualization of ER exit sites (ERES), using Sec31A as marker, did not indicate co-localization with IL-8 after archazolid treatment. However, calnexin, an ER-marker, co-localized with the extra-Golgi accumulation of IL-8 caused by archazolid. Note that neither in unstimulated nor in LPS-activated monocytes such co-localization of IL-8 and calnexin was evident, only in the presence of archazolid. Thus, we suggest that inhibition of V-ATPase by archazolid blocks secretion of de-novo-synthesized IL-8 at the ER.

We then investigated the ability of related tool/control compounds to cause accumulation of IL-8 at the ER. Of interest, those agents that increased the vesicular pH and caused enriched intracellular IL-8 such as NH₄Cl or monensin caused co-localization of IL-8 with the ER marker calnexin in analogy to archazolid, as did BFA. Chloroquine and CHX caused no co-localization of calnexin and IL-8, and also the pattern of IL-8 localization after BFA treatment differed somewhat from calnexin staining (**Fig. 6C**).

To confirm IL-8 accumulation at the ER by archazolid, we investigated the occurrence of ER stress (unfolded protein response, UPR) as consequence of blocked cytokine transport. ER stress is characterized by increased expression of BiP. Archazolid upregulated BiP expression after 24 h with maximal effects at 10 nM by about 3-fold. Other agents that blocked IL-8 secretion (i.e., NH₄Cl, monensin and BFA) also increased BiP expression, although to a varying extent with strong effects by BFA and monensin (15- and 8-fold) but rather moderate effects by NH₄Cl. Note that CQ (which failed to increase IL-8 at the ER) caused no elevation of BiP (**Fig. 6D**).

4. Discussion

Here we showed that archazolid represses the secretion of TNF- α , IL-6, IL-8, and IL-10 in LPS-stimulated primary human monocytes at effective concentrations of 10 nM. Our data indicate that archazolid causes retention of de-novo synthesized IL-8 (and TNF- α) at the ER associated with ER stress by inhibition of V-ATPase, without blocking IL-8 mRNA or protein expression. Comparative studies with apicularen and bafilomycin [30] imply that repression of cytokine release in activated monocytes is a class effect of V-ATPase inhibitors. Notably, central signaling pathways (i.e., p38 MAPK, Akt, ERK-1/2, SAPK/JNK, and I κ B) involved in LPS-induced monocyte activation, cytokine expression and secretion, as well as monocyte viability were unaffected by archazolid, indicating that the anti-inflammatory effect was achieved without a general cellular perturbation and V-ATPase inhibition was not leading to cytotoxicity in monocytes.

In contrast to monocytes, archazolid *in vitro* is instead well-documented to induce cell death in different cancer cells [15, 16, 18, 31] and its anti-tumoral activity *in vivo* has recently been reported [15, 32], supporting further investigation of this compound also on immune cells. In fact, besides cancer cells, the effects of archazolid on primary eukaryotic cells have not been reported yet, and studies, where other V-ATPase inhibitors have been analyzed in detail in monocytes are rare. The capacity of archazolid and other V-ATPase inhibitors to modulate cytokine release from activated primary human monocytes shown here, together with direct killing of cancer cells reported before, represents therefore a novel combined effect on two key elements of the tumor microenvironment, with therapeutic relevance in cancer treatment. Interestingly, recent studies have also shown that V-ATPase, particularly the $\alpha 2$ isoform ($\alpha 2V$) expressed on tumor cells, may have another role besides pH regulation, that is, generation of a pro-inflammatory peptide (called $\alpha 2NTD$) that activates monocytes [33, 34]. Together, these results clearly draw attention on V-ATPase as a key target to

manipulate the mutual communication between tumor cells and monocytes via cytokines and not only to suppress cancer cell viability.

The occurrence of V-ATPase in monocytic cells was demonstrated before [35], but the focus of V-ATPase research was placed on leukemia-derived cells lines (e.g., THP-1 and U937) [36] and on other differentiated cells of the monocyte/macrophage haematopoietic lineage including osteoclasts [37]. In fact, V-ATPase expression is increased during macrophage differentiation of THP-1 and U-937 [36]. We find that primary monocytes express membrane-associated V-ATPase that upon differential centrifugation co-localized with markers of the ER (calnexin) and lysosomes (LAMP1) but not with the Golgi (marker GM130). Also in our hands, V-ATPase protein was more abundant in the monocytic leukemia-derived cell line MM6 as compared to primary monocytes, supporting the hypothesis that hyperproliferative cells overexpress V-ATPase to maintain malignant growth [11, 12]. Despite lower protein levels, however, V-ATPase is expressed as functionally active enzyme in primary monocytes and archazolid or apicularen increased the vesicular pH. Similarly, in monocytic U937 cells, intracellular pH regulation by V-ATPase was shown by using bafilomycin as V-ATPase blocker [38].

Release of various cytokines is a major function of activated monocytes [1] and members of these secreted pro-inflammatory proteins, including TNF- α , IL-6 and IL-8, are key players in inflammation-triggered cancer [4, 5]. Archazolid caused suppression of LPS-induced TNF- α , IL-6, IL-8, and IL-10 release with significant effects at 10 nM. Note that the effectiveness of archazolid to repress TNF- α and IL-6 was limited to about 50% but still comparable to the effect of high dose (1 μ M) dexamethasone, used as control [39]. Apicularen and bafilomycin caused similar effects as archazolid suggesting a class effect of V-ATPase inhibitors. Also, IL-10 release was more efficiently blocked by archazolid as compared to dexamethasone. On the other hand, the LPS-induced release of IL-1 β was not inhibited but instead was enhanced by archazolid as well as by

apicularen and bafilomycin, while dexamethasone efficiently reduced its secretion, as reported [40]. IL-1 β possesses multiple and diverse properties in response to infections, injury, immune challenge, and it eventually ensures the recovery from trauma or infections, but also plays a pathogenic role in many chronic diseases, including diabetes, atherosclerosis and cancer [41]. Thus, an elevated IL-1 β release should be considered in this respect when applying V-ATPase inhibitor *in vivo*. Interestingly, IL-1 β lacks a secretory signal sequence and is, unlike TNF- α , IL-6, IL-8, and IL-10, secreted by alternative routes (not blocked by BFA or monensin) than the classical ER-Golgi pathway [42]. IL-1 β secretion may involve the externalization of the cytokine directly at the plasma membrane, via vesicles or microvesicles shed from the plasma membrane, and also exosomes may carry IL-1 β out of the cell [42]. Together, archazolid and dexamethasone seem to modulate different signaling pathways. In fact, archazolid failed to affect transcription of IL-8 in LPS-activated monocytes, in contrast to dexamethasone [43].

We found that the impaired extracellular release of IL-8 and TNF- α by V-ATPase inhibitors was accompanied by intracellular enrichment. BFA that inhibits secretion and fuses ER and Golgi [28, 44] also led to accumulation of intracellular IL-8 along with impaired amounts of released IL-8, and a similar effect was observed with the Na⁺/H⁺ antiporter monensin that blocks protein transport in the Golgi [29]. On the other hand, CHX that inhibits de-novo protein biosynthesis by blocking translational elongation [45] impaired the amounts of both extra- and intracellular IL-8. Conclusively, the reduced IL-8 release in LPS-stimulated monocytes by archazolid or apicularen was apparently due to a blockade of the secretion of de-novo synthesized IL-8 protein.

Bafilomycin impaired the transport between early and late endosomes by inhibiting acidification through V-ATPase inhibition [46]. Using NH₄Cl as tool compound to increase the pH in the secretory pathway, our data correlate organellar alkalization to repression of IL-8 release and intracellular accumulation of IL-8, respectively. Chloroquine only slightly increased the pH in

secretory organelles, IL-8 was not enriched in the cell, and BiP expression was not enhanced though extracellular IL-8 levels were lowered. This suggests that chloroquine affects expression of IL-8, supported by the finding that in LPS-stimulated monocytes chloroquine decreases IL-1 β and IL-6 mRNA and blocks maturation of TNF- α protein rather than blocking their secretion [47, 48].

Most cytokines including TNF- α , IL-6, IL-8, and IL-10 are released through classical secretion [49]. Thus, after cotranslational translocation into the ER, the cytokines are collected at the ERES, transported to the ERGIC from there through the Golgi to the TGN, where they are packaged in secretory vesicles and exocytosed through the plasma membrane (see Figure 6A). A recent study addressed IL-8 secretion in the monocytic cells line THP-1 [50] but its secretion from primary monocytes has not been investigated thus far. Our IF microscopic analysis applying select markers of ER, ERES, ERGIC, *cis-/medial*-Golgi, and *trans*-Golgi Network clearly demonstrate that upon archazolid treatment IL-8 accumulates in the ER. This is in agreement with the occurrence of the c subunit of V-ATPase in the ER compartment (i.e., the P13 fraction) upon differential centrifugation and with elevated BiP expression as marker of UPR/ER stress. Finally, also NH₄Cl and monensin led to co-localisation of IL-8 and calnexin and to increased BiP expression. The molecular reason for the ER-accumulation of IL-8 remains to be elucidated in future studies, but could be related to folding/maturation problems, recruitment/packaging in ERES or could be a backflow of sorting/transport problems further down the secretory pathway.

Taken together, our study revealed archazolid as efficient suppressor of classical secretion of pro- and anti-inflammatory cytokines in activated primary monocytes, seemingly due to an accumulation of the cargo within the ER that prevents cytokine transport. Because archazolid and related V-ATPase inhibitors are proposed to exhibit high potential as tumor therapeutics [11], the accompanied effect on the secretion of tumor-modulating cytokines may indeed affect tumor

growth and progression by modulating the tumor microenvironment, which should be taken into account in view of the clinical use as anti-cancer agents.

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Conflict of Interest Statement: None declared.

5. Literature

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Figure legends

Figure 1 Detection of V-ATPase protein in human PBMC and monocytes and in their subcellular fractions. (A) Protein levels of the c subunit of V-ATPase and of β -actin in whole cell lysates. Platelets, PBMC, monocytes, and MM6 cells were pelleted, lysed and equal amounts of protein (19 μ g) were analyzed. Data from densitometric analysis are means + SEM of fold V-ATPase/ β -actin ratio; n = 3. (B) Analysis of the c subunit of V-ATPase in supernatants (S100) and pellets (P100) after centrifugation of whole cell lysates of monocytes or MM6 cells at 100,000 $\times$ g. (C) Protein analysis in subcellular fractions of primary human monocytes. Whole cell lysates of primary human monocytes were centrifuged at 13 000 $\times$ g yielding pellet (P13) and supernatant (S13). The S13 was further centrifuged at 100 000 $\times$ g leading to supernatant (S100) and pellet (P100). Protein levels of the c subunit of V-ATPase, calnexin (ER marker), GM130 (*cis*-Golgi marker), LAMP1 (lysosomal marker), β -actin and β -tubulin in subcellular fractions were determined by Western blot. Pictures are representative of three independent experiments.

Figure 2. Vesicular and cytosolic pH and viability of primary human monocytes after archazolid treatment. (A) Monocytes (2.5×10^5 /mL) were incubated with archazolid (10 nM) or vehicle (DMSO) for 4 h. Acidic vesicles (red) were stained with the LysoTracker probe for 5 min. Fluorescence microscopy pictures are representative of four independent experiments. Scale bar: 10 μ m, contrast: differential interference contrast. (B) For determination of vesicular pH (left panel), monocytes were incubated with FITC-dextran (0.5 mg/mL), which is endocytosed and ends up in acidic vesicles. Cells were co-incubated with archazolid or apicularen at indicated concentrations for 4 h and the fluorescence at 520 nm was measured. Data correspond to the means + SEM; n = 7-8. Pictures show FITC-dextran uptake into monocytes. Green: FITC-dextran, blue: nuclei (DAPI). Scale bar: 10 μ m. Images are representative of two independent experiments. For determination of

the cytosolic pH (right panel), monocytes were incubated with archazolid or apicularen at the indicated concentrations for 4 h and stained with BCECF-AM (0.1 μ M). (C) Primary human monocytes (2×10^6 /mL) were pre-incubated with archazolid and apicularen at the indicated concentrations, with staurosporine (3 μ M) as positive control, and vehicle (DMSO) for 30 min. Cells were stimulated with LPS (10 ng/mL) or left unstimulated for 24 h. Cell viability was determined by MTT assay. Data are means + SEM; n = 6-8. ***P < 0.001 vs. the vehicle (DMSO); ANOVA + Bonferroni.

Figure 3. Effects of V-ATPase inhibitors on cytokine levels in supernatants and on signaling kinases of LPS-stimulated human monocytes. For analysis of cytokine release, primary human monocytes (1.5×10^6 /mL) were pre-incubated with archazolid, apicularen or bafilomycin at the indicated concentrations, with dexamethasone (Dex, 1 μ M), cycloheximide (CHX, 5 μ M), or vehicle (DMSO) for 30 min. Cells were then stimulated with LPS (10 ng/mL) for 4 h (TNF- α , IL-8) or 18 h (IL-6, IL-1 β , IL-10). Determination of cytokines in the supernatants was performed using ELISA. Data are expressed as percentage of control, means + SEM. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the vehicle (DMSO) stimulated with LPS; ANOVA + Bonferroni. (A) TNF- α : n = 4-6, 100% control: 5.28 ± 1.06 ng/mL; IL-6: n = 4, 100% control: 19.97 ± 5.16 ng/mL; IL-8: n = 4-8, 100% control: 12.48 ± 2.74 ng/mL; IL-10: n = 4, 100% control: 1.48 ± 0.51 ng/mL. (B) Bafilomycin: n = 3-5, 100% control: 6.68 ± 0.56 ng/mL; apicularen: n = 3, 100% control: 8.31 ± 2.82 ng/mL. (C) IL-1 β : archazolid: n = 5-6, 100% control: 5.46 ± 1.79 ng/mL; apicularen and bafilomycin: n = 3, 100% control: 9.19 ± 1.08 ng/mL. (D) Monocytes (1.25×10^6 /mL) were starved overnight, cells were

pre-incubated with test compounds at the indicated concentrations or with vehicle (DMSO) for 30 min, serum (2%, v/v) was added, and cells were stimulated with LPS (100 ng/mL) for 5 min (ERK-1/2, Akt, p38 MAPK) and 15 min (I κ B α , SAPK/JNK), respectively. Protein phosphorylation was analyzed by Western blot. Expression of the respective protein (regardless phosphorylation status) or β -actin was determined as control. Representative Western blots of three independent experiments are shown.

Figure 4. Archazolid does not affect IL-8 mRNA expression and causes intracellular accumulation of IL-8 protein in LPS-stimulated primary human monocytes. (A) IL-8 mRNA expression. Monocytes (1.5×10^6 /mL) were pre-incubated with archazolid (10 nM) or vehicle (DMSO) for 30 min and stimulated with LPS (10 ng/mL) for indicated times. RNA was isolated and relative IL-8 mRNA expression was determined using taqman probes. Normalization to the housekeeping gene GAPDH was performed. Data are expressed as mean $\pm$ SEM, n = 3. (B, C) Analysis of intracellular (white bars) and extracellular levels (black bars) of IL-8 protein. Monocytes (1.5×10^6 /mL) were pre-incubated with archazolid (arch), bafilomycin (baf), apicularen (apic), monensin (mon), brefeldin (BFA), cycloheximide (CHX) or vehicle (DMSO) for 30 min. Cells were stimulated with LPS (10 ng/mL) for 4 h. Supernatants were collected and cells were lysed. Cytokine concentrations in supernatants (extracellular) and cell lysates (intracellular) were determined using ELISA. Data are expressed as percentage of control, means + SEM. (B) ANOVA + Bonferroni of log transformed data; n = 5-10; **P < 0.01, ***P < 0.001 vs. the vehicle (DMSO)

stimulated with LPS, 100% control: 2.53 ± 0.58 ng. (C) Paired t test; $n = 5$; $**P < 0.01$, $***P < 0.001$ vs vehicle (DMSO for monensin and ethanol for BFA) stimulated with LPS; 100% control (DMSO): 5.91 ± 1.28 ng, 100% control (ethanol): 5.26 ± 1.14 ng. (D) Immunofluorescence microscopic analysis of IL-8 protein. Monocytes were incubated as described for (B) and (C). After formaldehyde fixation, IL-8 protein was detected by anti-IL-8 antibody and visualized using Alexa Fluor 555 conjugated secondary antibody. Green: IL-8 (IL-8 staining was changed from red to green for clarity and uniform illustration); blue: nuclei (DAPI); scale bar: 5 μ m. Pictures are representative for four independent experiments.

Figure 5. Increase of vesicular pH affects intracellular and extracellular IL-8 levels of human monocytes. (A) Effects of NH_4Cl , chloroquine (CQ), and archazolid on vesicular pH. Test compounds were added to monocytes for 4 hrs and vesicular pH was determined as described in the legend to Fig. 2B. Data are means + SEM, $n = 3-8$. (B) Effects of NH_4Cl , chloroquine and archazolid on extracellular and intracellular IL-8 protein levels in monocytes. IL-8 concentrations were determined by ELISA as described in legend to Fig. 4B/C. Data are expressed as percentage of control, means + SEM, $n = 3-10$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs 100% control; ANOVA + Bonferroni, NH_4Cl (50 mM): paired t test.

Figure 6. Visualization of intracellular IL-8 in human monocytes and analysis of ER stress.

(A) Schematic representation of the classical exocytotic pathway via ER and Golgi; reproduced

according to [50]. (B, C) Co-localisation of IL-8 with various cell organelle markers in LPS-stimulated monocytes. Monocytes were pre-incubated with archazolid (10 nM), NH₄Cl (20 mM or 50 mM), chloroquine (CQ, 100 μ M), BFA (1 μ g/mL), monensin (Mon, 10 μ M) and cycloheximide (CHX, 5 μ M) as negative control for 30 min and stimulated with LPS (10 ng/mL) or left untreated for 4 h. After fixation, cells were incubated with antibodies against marker proteins and IL-8, as well as with fluorophore-labeled secondary antibodies for signal visualization. Marker proteins, red: *trans*-Golgi, TGN38; *cis*- and *medial*-Golgi, giantin; ER-Golgi intermediate compartment, ERGIC-53; ER exit sites, Sec31A; ER, calnexin. Green: IL-8. Blue: nuclei (DAPI). Scale bar: 5 μ m. Pictures are representative for three independent experiments. (D) Expression of BiP as marker for ER stress. Monocytes were incubated with archazolid, NH₄Cl, chloroquine (CQ), monensin or BFA at the indicated concentrations. After 24 hrs at 37°C cells were lysed and the amounts of BiP and β -actin were determined by Western blot and densitometric analysis. Blots are representative for three independent experiments and data from densitometric analysis are means + SEM of fold BiP/ β -actin ratio; n = 4-5.