Pro-inflammatory cytokines enhance dilatation of bile canaliculi caused by cholestatic antibiotics

Ahmad Sharanek, Audrey Burban, Nadia Ciriaci, André Guillouzo

Pro-inflammatory cytokines enhance dilatation of bile canaliculi caused by cholestatic antibiotics

Ahmad Sharanek[1], Audrey Burban1, Nadia Ciriaci and André Guillouzo[*]
andre.guillouzo@univ-rennes1.fr

Univ Rennes, Inserm, Inra, Institut NUMECAN (Nutrition Metabolisms and Cancer), UMR_S 1241, 35000 Rennes, France

[*]Corresponding author at: André Guillouzo, Inserm UMR 1241, Numecan, Faculté des Sciences Pharmaceutiques et Biologiques, 35043 Rennes Cedex, France.

[1] Both authors contributed equally to this work.

Abstract

Many drugs can induce liver injury, characterized by hepatocellular, cholestatic or mixed hepatocellular-cholestatic lesions**.** While an inflammatory stress is known to aggravate hepatocellular injury caused by some drugs much less evidence exists for cholestatic features. In this study, the influence of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α), either individually or combined, on cytotoxic and cholestatic properties of antibiotics was evaluated using differentiated HepaRG cells. Six antibiotics of various chemical structures and known to cause cholestasis and/or hepatocellular injury in clinic were investigated. Caspase-3 activity was increased with all these tested hepatotoxic drugs and except with erythromycin, was further augmented in presence of cytokines mainly when these were co-added as a mixture. TNF-α and IL-1β aggravated cytotoxicity of TVX more than IL-6. Bile canaliculi (BC) dilatation induced by cholestatic drugs was increased by co-treatment with IL-6 and IL-1β but not with TNF-α. Reduced accumulation of carboxy-dichlorofluorescein, a substrate of the multi-drug resistance-associated protein 2, in antibiotic–induced dilatated BC, was further extended in presence of individual or mixed cytokines. In conclusion, our data demonstrate that pro-inflammatory cytokines either individually or in mixture, can modulate cholestatic and/or cytotoxic responses to antibiotics and that the extent of these effects is dependent on the cytokine and the cholestatic antibiotic.

Keywords: Drug-induced liver injury, cholestasis; HepaRG cells; inflammatory stress, canalicular efflux, bile canaliculi

Abbreviations

AMP ampicillin

ATB antibiotic

BA bile acid

BC bile canaliculi

BSEP bile salt export pump

CDF 5 (and 6)-carboxy-2′,7′-dichlorofluorescein

CDFDA 5(6)-carboxy-2, 7-dichlorofluorescein diacetate (CDFDA)

CLX cloxacillin

CRP C-reactive protein

DMSO dimethyl sulfoxide

ERY erythromycin

FLX flucloxacilin

IL interleukin

LPS lipopolysaccharide

LVX levofloxacin

MTT methylthiazol tetrazolium

MRP2 multi-drug resistance-associated protein 2

NAF nafcillin

PRA penicillinase-resistant antibiotic

NTCP Na+-dependent taurocholic cotransporting polypeptide

SM streptomycin

TNF-α tumor necrosis factor-α

TVX trovafloxacin.

Introduction

Liver lesions are usually categorized into hepatocellular, cholestatic, or mixed (cholestatic and hepatocellular) injury based on the pattern of liver enzyme abnormalities. Cholestasis is characterized by accumulation of bile acids (BAs) or their conjugated bile salts in the liver and systemic circulation. Both intrahepatic and extrahepatic cholestasis has been described in humans and may be due to impaired secretion of BAs by hepatocytes or obstruction of either intrahepatic or extrahepatic bile ducts.

Cholestasis is an early and frequent complication in patients with sepsis that is characterized by a systemic inappropriate inflammatory response to bacterial infection resulting in the release of lipopolysaccharide (LPS) and production of pro-inflammatory cytokines and other mediators (Chand and Sanyal, 2007) . LPS and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6), are potent inhibitors of hepatobiliary transport. Expression and activities of key sinusoidal and canalicular transporters of hepatocytes are reduced and both bile acid-dependent and bile acid-independent fractions of bile flow are affected (Elferink et al., 2004; Fardel and Le Vee, 2009). Increased levels of intrahepatic BAs have been shown to promote inflammation in cholestasis associated with LPS-induced sepsis in mice (Hao et al., 2017).

Idiosyncratic bland and mixed cholestasis has been observed in clinic following treatment with many drugs, including various antibiotics (ATBs). The mechanisms underlying idiosyncratic drug-induced liver injury remain poorly understood (Shaw et al., 2010). One hypothesis is that an inflammatory stress alters the toxicity threshold of an individual, rendering toxic a therapeutic dose of a drug (Ganey et al., 2004). The most prescribed antibiotic flucloxacilin (FLX) is thought to cause liver damage,

mostly cholestasis, in approximately 8.5 in 100000 patients (Russmann et al., 2005). Recently, we reported that FLX and two other penicillinase-resistant antibiotics (PRAs), namely cloxacillin (CLX) and nafcillin (NAF), induced cholestatic features at much lower concentrations than cytotoxicity in human HepaRG cells while other hepatotoxic ATBs such as trovafloxacin (TVX), levofloxacin (LVX) and erythromycin (ERY), induced cytotoxicity and cholestasis features at comparable concentrations (Burban et al., 2017). All these compounds caused dilatation of BC. However, whether these drug-induced cholestatic features can be aggravated in an inflammatory context in absence of BA accumulation, remains unclear. As a first approach, the present study aimed to investigate whether an inflammatory stress could modulate deformation of BC and related disturbances induced by these cholestatic drugs using differentiated HepaRG cells. The major pro-inflammatory cytokines TNF-α, IL-6 and IL-1β, either individually or combined, were selected to create an inflammatory situation before simultaneous treatment with the tested ATBs. Our data demonstrate that pro-inflammatory cytokines can aggravate ATB-induced cholestatic and cytotoxic effects.

Materials and Methods

Reagents

Flucloxacillin (FLX), cloxacillin (CLX), nafcillin (NAF), trovafloxacin (TVX), levofloxacin (LVX), erythromycin (ERY), streptomycin (SM), ampicillin (AMP), methylthiazol tetrazolium (MTT), N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) and 5(6)-carboxy-2, 7-dichlorofluorescein diacetate (CDFDA) were purchased from Sigma (St. Quentin Fallavier, France). [^{3}H]-Taurocholic acid ([^{3}H]-TA) was from Perkin Elmer (Boston, MA). TNF-α, IL-6 and IL-

1β were provided by Promocell (Nuremberg, Germany). Other chemicals were of reagent grade.

Cell cultures

All HepaRG cell cultures were prepared from vials of different frozen cell batches and incubated with the same HyClone fetal calf serum. Before starting the treatments HepaRG cells were differentiated as previously described (Cerec et al., 2007). Cells were seeded at a density of 2.6×10^4 cells/cm^2 in Williams' E medium supplemented with 2 mM glutamax, 100 U/mL penicillin, 100 µg/mL streptomycin, 10% HyClone fetal calf serum, 5 µg/mL insulin, and 50 µM hydrocortisone hemisuccinate. After 2 weeks, they were shifted to the same medium supplemented with 1.7% dimethyl sulfoxide (DMSO) for 2 additional weeks to obtain differentiated cultures containing around equal proportions of hepatocyte-like and progenitor/primitive biliary-like cells.

Cell treatments

Differentiated HepaRG cells were first incubated in absence or presence of IL-6, IL-1β and TNF-α, either individually or combined, in a medium containing 2% HyClone fetal calf serum and 1% DMSO (final concentration) for 24 h before simultaneous incubation with ATBs for 2 or 24 h in the same medium.

C-reactive protein ELISA assay

C-reactive protein (CRP) was measured in culture media using the CRP DuoSet kit (Catalog Number: DY1707, R&D, Abingdon, United Kingdom), according to manufacturer's instructions. Briefly, media were collected after 24 h treatment and

stored at −80 °C until analysis; 96-well microplates were coated with mouse anti-human CRP capture antibody and incubated overnight. A standard curve was obtained by using recombinant human CRP at 2-fold serial dilutions from 1000 pg/mL to 15.6 pg/mL, as recommended by the manufacturer. Samples were diluted appropriately and added for 2 h after a saturation step. Biotinylated mouse anti-human CRP antibody was added for 2 h after washing. Streptavidin-horseradish peroxidase and its substrate were used for the revelation step. Optical density was read at 450 nm with wavelength correction.

Caspase-3 activity determination

After treatment, cells were scrapped in their culture medium to also collect any detached cells, centrifuged, washed with phosphate buffered saline and stored as pellets at −80 °C. After cell lysis, the Bradford protein assay was used to measure the concentration of total protein in each sample and 40 µg proteins were incubated with 50 µM Ac-DEVD-AMC in caspase 3 activity buffer (20 mM PIPES pH 7.2, 100 mM NaCl, 10 mM dithiotreitol, 1 mM EDTA, 0.1% CHAPS and 10% sucrose) at 37 °C for 1 h. Caspase-3-mediated cleavage of the Ac-DEVD-AMC peptide was continuously measured by spectrofluorimetry using excitation/emission wavelengths of 380/440 nm (Burban et al., 2017).

Cell viability assay

Cytotoxicity was evaluated by the MTT colorimetric assay. Briefly, the cells were seeded in 96-well plates and treated with various concentrations of ATBs in triplicate for 24 h. After medium removal, 100 µl serum-free medium containing MTT (0.5 mg/mL) was added to each well and incubated for 2 h at 37 °C. The water-insoluble formazan was dissolved in 100 µl DMSO, and absorbance was measured at 550 nm.

Time-lapse cell imaging

Phase-contrast images of HepaRG cells were captured by time-lapse phase-contrast videomicroscopy equipped with an AxioCam MRm camera. An inverted microscope (Zeiss Axiovert 200 M), equipped with a thermostatic chamber (37 °C and 5% CO_2), was used to maintain the cells under normal culture conditions (Sharanek et al., 2016).

Bile canaliculi area quantification

Bile canaliculi (BC) area quantification was based on phase-contrast images. After capturing of images, BC areas were determined from 9 zones per condition counting around 2500 objects in 3 independent experiments using ImageJ 1.48 software after different times of exposure. Bright objects corresponding to BC were segmented by adjusting the shape and area parameters to exclude non-corresponding objects (Sharanek et al., 2016).

Taurocholic acid uptake

HepaRG cells were treated with cytokines or ATBs separately or simultaneously for 24 h and then washed with a standard buffer and incubated with [^{3}H]-TCA for 30 min in presence and absence of Na+, to evaluate Na+-dependent taurocholic cotransporting polypeptide (NTCP) activity. After 30 min incubation, cells were washed twice with phosphate buffered saline and lysed with 0.1N NaOH. Accumulation of radiolabeled substrate was determined through scintillation counting. Taurocholate accumulation values in presence of sodium minus accumulation values in absence of sodium represented NTCP activity (Antherieu et al., 2013). Data are the mean ± SEM of 3 independent experiments. Results are expressed relative to the

levels found in untreated (control) cells, arbitrarily set at 100%. *$p < 0.05$ compared with untreated cells.

Taurocholic acid efflux

TCA is mainly effluxed by the bile salt export pump (BSEP). To investigate the effects of ATBs and cytokines on TCA efflux, cell cultures were washed with a standard buffer containing Ca^{2+} and Mg^{2+}, exposed to 43.3 nM [^{3}H]-TCA for 30 min to induce its intracellular accumulation, washed and then incubated with ATBs and/or cytokines for 2 h. All these steps were performed using the standard buffer. After incubation, cells were washed and scraped in 0.1 N NaOH and the remaining radiolabeled substrate was measured through scintillation. [^{3}H]-TCA efflux was determined based on its accumulation in cell layers (cells + BC) and calculated relative to the controls using the following formula: [^{3}H]-TCA efflux = [^{3}H]-TCA accumulation in cell layers *Control* / [^{3}H]-TCA accumulation in cell layers *Tested compound* *100 (Sharanek et al., 2016).

Carboxy-dichlorofluorescein efflux

After treatment cells were washed with warm Williams' E medium without phenol red and any supplement, and incubated with the same medium containing 3 µM CDFDA for 30 min at 37 °C. CDFDA passively crossed the plasma membrane and upon hydrolysis by intracellular esterases, was converted to fluorescent CDF (excitation/emission: 488/509 nm) and directed towards the biliary pole by membrane transporters, particularly the multi-drug resistance associated protein 2 (MRP2). After washing, imaging was performed using a Cellomics ArrayScan VTI

HCS Reader (Thermo Fisher Scientific). The number of CDF-accumulating BC was quantified using ImageJ 1.48 software (Burban et al., 2017).

Statistical analysis

A one-way ANOVA with a multiple comparison test (GraphPad Prism 5.00) was performed to compare the data. Each value corresponded to the mean ± SEM of three independent experiments. Data were considered significantly different at $p < 0.05$.

Results

Effects of pro-inflammatory cytokines on antibiotic-induced cytotoxicity in HepaRG cells

First, we investigated cytotoxic effects of the three tested pro-inflammatory cytokines, TNF-α, IL-6, and IL-1β, either individually or combined, in HepaRG cells. At concentrations that have been widely used in *in vitro* studies, i.e. TNF-α (10 ng/ml), IL-6 (5 ng/ml) and IL-1β (0.5 ng/ml) (Al-Attrache et al., 2016; Bachour-El Azzi et al., 2014; Rubin et al., 2015), none of these cytokines caused any cytotoxicity**,** as evaluated by caspase-3 activity and MTT assays after 2 daily repeated additions. Similarly, when added as a mixture the 3 pro-inflammatory cytokines did not exert any significant effect (Figure 1A). Generation of an *in vitro* inflammatory response to these cytokines at the tested concentrations was validated by determination of secreted CRP levels after 24 h incubation. All cytokines caused statistically significant increase in secreted CRP amounts, reaching 3-, 14- and 17-fold with TNF-α, IL-6 and IL-1β respectively, compared with unexposed cell cultures; treatment with

the cytokine mixture further enhanced CRP secretion levels reaching 37-fold (Figure 1B).

Cytotoxicity of six cholestatic (FLX, CLX, NAF, TVX, LVX and ERY) and two non cholestatic (AMP and SM) antibiotics was also estimated in HepaRG cells using caspase-3 activity assay after 24 h treatment. To verify whether ATB-induced apoptotic effects were affected by co-treatment with cytokines, HepaRG cells were first incubated with TNF-α, IL-6 and IL-1β either individually or combined before simultaneous co-treatment with the tested ATBs for additional 24 h. As previously reported (Burban et al., 2018), the tested hepatotoxic ATBs induced dose-dependent enhancement of caspase-3 activity. NAF was slightly more cytotoxic than the two other PRAs. When co-added with PRAs, individual cytokines were ineffective while their mixture enhanced around 2-fold caspase-3 activity levels compared to the values measured with PRAs alone (Figure 2 A-C).

At 5 µM TVX did not augment caspase-3 activity whereas its co-addition with either of the three cytokines caused around 3-fold increase. At 20 and 100 µM TVX co-added with TNF-α, IL-6 and IL-1β , further enhanced caspase-3 activity, reaching 23-, 14.6- and 26-fold respectively with the highest concentration compared to 8.6-fold when added alone. Notably, caspase-3 activity elevation reached 36-fold when 100 µM TVX was co-added with the cytokine mixture (Figure 2D). LVX alone had no significant effects at concentrations up to 2.5 mM, in the presence of individual cytokines; by contrast, at this high concentration caspase-3 activity was enhanced 5.5-fold in presence of the cytokine mixture (Figure 2E).

ERY also augmented dose-dependently caspase 3 activity, reaching around 4- and 12.8-fold at 0.5 and 1 mM respectively after 24 h; while individual cytokines were

ineffective their mixture reduced ERY-induced activity to around 2.5- and 5-fold respectively (Figure 2F).

The two non cholestatic non hepatotoxic ATBs SM and AMP were used as controls: SM was slightly cytotoxic, causing 2.2-fold induction of caspase-3 activity only at 12 mM while AMP had no effect at concentrations up to 24 mM. Co-treatment with the cytokines, either individually or in mixture, was ineffective regardless of the concentration of these two ATBs (Figure 2G and H).

Cytotoxicity of 5 ATBs in presence or absence of the 3 cytokines was also estimated using the MTT assay; only aggravation of TVX toxicity by TNF-α was observed (Supplemental Figure 1).

Effects of inflammatory stress on ATB-induced cholestatic features

Based on a previous study (Burban et al., 2018), ATBs were used at the following concentrations: 2 mM FLX, 2 mM CLX, 2 mM NAF, 20 µM TVX, 0.5 mM LVX, 0.1 mM ERY, 12 mM SM and 24 mM AMP. At these concentrations none of the tested ATBs was significantly cytotoxic even when co-incubated with cytokines (Figure 2). No increase in caspase 3 activity and no morphological changes, except BC dilatation with cholestatic ATBs, were evidenced. BC deformation, an important feature of drug-induced cholestasis (Sharanek et al., 2016), was analyzed during 24 h following addition of ATBs, either alone or in presence of individual or combined cytokines, using time-lapse microscopy. All tested cholestatic ATBs caused BC dilatation as early as 2 h treatment. PRAs induced higher dilatation of BC than TVX, LVX and ERY (Figure 3A and B). The negative controls SM and AMP were ineffective (Figure 3A and C). The three cytokines, either individually or in mixture, did not alter BC morphology (Figure 3A and D). However, co-treatment with IL-6 and

IL-1β but not with TNF-α, significantly aggravated BC dilatation induced by all tested cholestatic ATBs. No further aggravation was observed with the cytokine mixture (Figure 3A and B).

As BC deformations could be associated with bile flow failure, we analyzed whether cholestatic ATBs impaired efflux activity by measuring TCA accumulation in cell layers (intracelllular + canalicular accumulation) and canalicular accumulation of the fluorescent probe CDF. As previously reported cytokines inhibited BA uptake via inhibition of the NTCP transporter (Bachour-El Azzi et al., 2014; Le Vee et al., 2009). In our experimental conditions TNF-α, IL-6 and IL-1β repressed NTCP activity by 40 to 75% after 24h exposure (Figure 4A), indicating that TCA accumulation in cell layers could not be correctly analyzed after 24 h pre-incubation with pro-inflammatory cytokines. Consequently, HepaRG cells were directly treated with cytokines and ATBs either separately or combined. Inhibition of [^{3}H]-TCA efflux was observed with the 6 cholestatic drugs, especially FLX (80%) and NAF (65%). At the tested concentrations the 3 cytokines showed only a trend to accumulation of TCA in cell layers and did not enhance TCA accumulation induced by the cholestatic drugs (Figure 4B and C).

After 2 h CDFDA addition, fluorescent CDF was well visualized in BC lumen of untreated and ATB-treated HepaRG cells (Figure 5A). Even though either individually or combined cytokines did not modulate CDF canalicular efflux (Figure 5D), their co-treatment with cholestatic ATBs resulted in significant reduction of CDF canalicular accumulation reaching between 40% and 80% (Figure 5A and B). The significant inhibition of CDF canalicular efflux by PRAs at 2 mM was aggravated in presence of pro-inflammatory cytokines (Supplemental Figure 2). To better demonstrate effects of cytokine co-addition, PRAs were used at a lower concentration (0.5 mM) (Figure 5A

and B). TNF-α caused the lowest effects. No further reduction of CDF accumulation was observed when the cells were co-treated with mixed versus individual cytokines. SM and AMP were ineffective, even in presence of individual or combined cytokines (Figure 5A and C).

Discussion

Several reports have shown that modest activation of the innate immune system by inflammagens such as LPS, can markedly enhance hepatotoxic response to various drugs, such as amiodarone, diclofenac and TVX (Roth et al., 2017). However, influence of an inflammatory stress on drug-induced liver injury has mostly been focused on cytotoxic damage, cholestatic effects remaining poorly investigated. A direct inflammatory response to LPS (Aninat et al., 2008) and pro-inflammatory cytokines (Bachour-El Azzi et al., 2014; Klein et al., 2015; Rubin et al., 2015) has also been evidenced in HepaRG cells. In the current work using these cells, we showed that pro-inflammatory cytokines enhanced cholestatic features and/or cytotoxic damage caused by ATBs exhibiting various chemical structures and known to induce liver injury in some patients.

TVX was the most potent cytotoxic drug among the tested ATBs and its cytotoxicity was potentiated by TNF-α and IL-1β. In agreement, these two cytokines have been reported to be predominantly involved in drug-cytokines synergies (Cosgrove et al., 2009; Fredriksson et al., 2014). High sensitivity to TVX in an inflammatory context is supported by several previous works using *in vitro* and *in vivo* models and has been related to C-Jun N-terminal kinase activation (Beggs et al., 2014; Maiuri et al., 2015). Contrary to TVX, the other fluoroquinolone LVX is generally considered a safe

antibiotic although rare cases of hepatotoxicity have been reported in clinic (Levine et al., 2014). Accordingly, this drug has to be used at high concentrations to cause cytotoxic damage in HepaRG cells (Burban et al., 2018) and contrary to TVX, it was not more cytotoxic with TNF-α than with other cytokines, supporting previous observations (Cosgrove et al., 2009).

As other macrolide ATBs, ERY has been shown to form P450-Fe^{2+}-metabolite complexes after CYP3A4-mediated activation (Larrey et al., 1983; Yamazaki et al., 1996). In the present study, ERY was found to be less cytotoxic when co-added with the cytokine mixture while individual cytokines were ineffective. The effect of the mixture could result from a faster decrease in the formation of reactive metabolites, related to early higher inhibition of CYP3A4 activity and/or to a stronger activation of defense mechanisms. Further work is required to clarify this point.

LPS and pro-inflammatory cytokines TNF-α, IL-1β and IL-6 can stimulate hepatocyte signaling responses through activation of a diversity of intracellular signal transduction pathways, such as MAPKs and JNK (Fardel and Le Vee, 2009). As previously reported (Burban et al., 2017), all presently tested hepatotoxic ATBs induced ER stress followed by oxidative stress. Whether the cytokines aggravated cytotoxic and cholestatic effects through these stress pathways required further investigation.

As recently observed (Burban et al., 2018), all tested cholestatic ATBs caused early BC dilatation and other associated cholestatic features in absence of cytotoxicity. Since cholestasis is an early and common event associated with sepsis, it was tempting to postulate that cholestatic features induced by cholestatic drugs could be aggravated in human hepatocytes cultured in an inflammatory context. Our data demonstrate that, although they had no effect on BC morphology when added alone,

IL-6 and IL-1β but not TNF-α aggravated BC dilatation caused by all cholestatic ATBs in HepaRG hepatocytes. Noticeably, TNF-α was also found to be ineffective on diclofenac-induced BC deformation (Al-Attrache et al., 2016). Importantly, cytokine effects were evaluated in a medium containing 2% bovine serum (i.e. containing only around 365 nM BAs) (Sharanek et al., 2015). Consequently, whatever the experimental condition no BA intracellular accumulation could occur.

Cholestasis is associated with impaired bile flow and several studies have shown that pro-inflammatory cytokines can alter expression and/or activity of various sinusoidal and canalicular transporters (Diao et al., 2010; Elferink et al., 2004). In the present study, none of the tested cytokines was found to markedly alter efflux of TCA, a BSEP substrate, and efflux of CDF, a MRP2 substrate. Moreover, they did not enhance TCA accumulation in cell layers induced by ATBs, likely resulting from BSEP inhibition. However they further reduced CDF canalicular accumulation observed with ATBs. Noticeably, discrepant data have been reported on the effects of pro-inflammatory cytokines on these two main canalicular transporters as well as other transporters (Bachour-El Azzi et al., 2014; Diao et al., 2010; Le Vee et al., 2009). They could be explained by the use of different experimental culture conditions as well as the choice of the parameter tested, e.g. expression or activity of the tested transporters, concentrations of cytokines and drugs and duration of treatment. However, the 3 cytokines were found to repress NTCP in agreement with other studies (Fardel and Le Vee, 2009), and to cause significant inhibition of CDF canalicular accumulation even in the presence of ATB concentrations that did not modulate CDF efflux activity. Whether this could be explained by further inhibition of transporters, some diffusion through disrupted tight-junctions or another mechanism, warrants further investigation.

Importantly, culture conditions could also influence the response of HepaRG cells to pro-inflammatory cytokines. Indeed, these cells were incubated in a medium containing 2% serum and 1% DMSO that certainly had some protective effects. Moreover, HepaRG cells have a high detoxifying capacity (Guillouzo and Guguen-Guillouzo, 2018). Therefore, it might be postulated that cytokine effects could be amplified by incubating the cells in serum- and DMSO-free medium. However, previous studies have shown that in presence of 2% serum limited changes if any were observed in drug biokinetics in HepaRG cells (Broeders et al., 2015; Pomponio et al., 2015).

Molecular mechanisms as to how BAs initiate liver injury are not completely elucidated. Cholestatic liver injury has been first associated with accumulation of toxic BAs resulting in mitochondrial dysfunction and cell death (Galle et al., 1990). However, much lower BA concentrations were found to induce toxic damage *in vivo* compared to in *vitro*, suggesting the involvement of sterile inflammation and innate immunity in the initial injury (Woolbright and Jaeschke, 2012). Recent studies have shown that at high concentrations BAs triggered early hepatocyte production of cytokines of which release enhanced neutrophil chemotaxis and liver infiltration in mice (Cai et al., 2017; Woolbright and Jaeschke, 2016). These findings were supported by the observation that periportal neutrophil infiltration correlated with serum alanine aminotransferase levels in cholestatic patients (Cai et al., 2017).

Cholestatic drugs can cause rapid cellular accumulation of toxic hydrophobic BAs in HepaRG cells in absence of major cellular injury when exogenous BAs are added to the culture medium (Burban et al., 2019; Sharanek et al., 2017). In the current study we show that at the tested concentrations the two pro-inflammatory cytokines IL6 and IL-1β enhanced BC dilatation induced by cholestatic ATBs in absence of obvious cell

injury. *In vivo,* cholestatic ATBs and other drugs can cause bland (e.g. FLX) or mixed (e.g. TVX) cholestasis, suggesting that in addition to environmental factors drug related factors are important in the occurrence of cholestasis implicating different mechanisms and activation of diverse signaling pathways. Further investigations on interactions between BAs, pro-inflammatory cytokines and/or immune cells and cholestatic drugs using different experimental conditions should permit to better understand the role of an inflammatory context in the potential of certain drugs to induce cholestasis.

In conclusion, our results demonstrate that certain pro-inflammatory cytokines can aggravate BC dilatation and CDF efflux inhibition induced by cholestatic antibiotics in HepaRG hepatocytes and that these effects can be observed in absence of cellular damage, suggesting that cholestatic features can be more sensitive than cellular injury to an inflammatory stress.

Acknowledgments

This work was partly supported by the European Community through the Innovative Medicines Initiative Joint Undertaking MIP-DILI project [grant agreement number 115336], resources of which are composed of financial contribution from the European Union's Seventh Framework Programme [FP7/20072013] and EFPIA companies' in kind contribution. Ahmad Sharanek and Audrey Burban were financially supported by the MIP-DILI project. Nadia Ciriaci was supported by a short-term fellowship from the National Council of Scientific and Technical Research (CONICET) and National University of Rosario. Permanent address: Institute of Experimental Physiology (IFISE), CONICET, Rosario, Argentina. We are grateful to Dr Rémy Le Guevel from the ImPACcell platform for image acquisition.

References

Al-Attrache, H., Sharanek, A., Burban, A., Burbank, M., Gicquel, T., Abdel-Razzak, Z., Guguen-Guillouzo, C., Morel, I., Guillouzo, A., 2016. Differential sensitivity of metabolically competent and non-competent HepaRG cells to apoptosis induced by diclofenac combined or not with TNF-alpha. Toxicology letters 258, 71-86.

Aninat, C., Seguin, P., Descheemaeker, P.N., Morel, F., Malledant, Y., Guillouzo, A., 2008. Catecholamines induce an inflammatory response in human hepatocytes. Crit Care Med 36, 848-854.

Antherieu, S., Bachour-El Azzi, P., Dumont, J., Abdel-Razzak, Z., Guguen-Guillouzo, C., Fromenty, B., Robin, M.A., Guillouzo, A., 2013. Oxidative stress plays a major role in chlorpromazine-induced cholestasis in human HepaRG cells. Hepatology 57, 1518-1529.

Bachour-El Azzi, P., Sharanek, A., Abdel-Razzak, Z., Antherieu, S., Al-Attrache, H., Savary, C.C., Lepage, S., Morel, I., Labbe, G., Guguen-Guillouzo, C., Guillouzo, A., 2014. Impact of inflammation on chlorpromazine-induced cytotoxicity and cholestatic features in HepaRG cells. Drug metabolism and disposition: the biological fate of chemicals 42, 1556-1566.

Beggs, K.M., Fullerton, A.M., Miyakawa, K., Ganey, P.E., Roth, R.A., 2014. Molecular mechanisms of hepatocellular apoptosis induced by trovafloxacin-tumor necrosis factor-alpha interaction. Toxicol Sci 137, 91-101.

Broeders, J.J., Parmentier, C., Truisi, G.L., Josse, R., Alexandre, E., Savary, C.C., Hewitt, P.G., Mueller, S.O., Guillouzo, A., Richert, L., van Eijkeren, J.C., Hermens, J.L., Blaauboer, B.J., 2015. Biokinetics of chlorpromazine in primary rat and human hepatocytes and human HepaRG cells after repeated exposure. Toxicology in vitro : an international journal published in association with BIBRA 30, 52-61.

Burban, A., Sharanek, A., Guguen-Guillouzo, C., Guillouzo, A., 2018. Endoplasmic reticulum stress precedes oxidative stress in antibiotic-induced cholestasis and cytotoxicity in human hepatocytes. Free radical biology & medicine 115, 166-178.

Burban, A., Sharanek, A., Hue, R., Gay, M., Routier, S., Guillouzo, A., Guguen-Guillouzo, C., 2017. Penicillinase-resistant antibiotics induce non-immune-mediated cholestasis through HSP27 activation associated with PKC/P38 and PI3K/AKT signaling pathways. Scientific reports 7, 1815.

Burban, A., Sharanek, A., Humbert, L., Eguether, T., Guguen-Guillouzo, C., Rainteau, D., Guillouzo, A., 2019. Predictive Value of Cellular Accumulation of Hydrophobic Bile Acids as a Marker of Cholestatic Drug Potential. Toxicological sciences : an official journal of the Society of Toxicology.

Cai, S.Y., Ouyang, X., Chen, Y., Soroka, C.J., Wang, J., Mennone, A., Wang, Y., Mehal, W.Z., Jain, D., Boyer, J.L., 2017. Bile acids initiate cholestatic liver injury by triggering a hepatocyte-specific inflammatory response. JCI insight 2, e90780.

Cerec, V., Glaise, D., Garnier, D., Morosan, S., Turlin, B., Drenou, B., Gripon, P., Kremsdorf, D., Guguen-Guillouzo, C., Corlu, A., 2007. Transdifferentiation of hepatocyte-like cells from the human hepatoma HepaRG cell line through bipotent progenitor. Hepatology 45, 957-967.

Chand, N., Sanyal, A.J., 2007. Sepsis-induced cholestasis. Hepatology 45, 230-241.

Cosgrove, B.D., King, B.M., Hasan, M.A., Alexopoulos, L.G., Farazi, P.A., Hendriks, B.S., Griffith, L.G., Sorger, P.K., Tidor, B., Xu, J.J., Lauffenburger, D.A., 2009. Synergistic drug-cytokine induction of hepatocellular death as an in vitro approach for the study of inflammation-associated idiosyncratic drug hepatotoxicity. Toxicology and applied pharmacology 237, 317-330.

Diao, L., Li, N., Brayman, T.G., Hotz, K.J., Lai, Y., 2010. Regulation of MRP2/ABCC2 and BSEP/ABCB11 expression in sandwich cultured human and rat hepatocytes exposed to inflammatory cytokines TNF-{alpha}, IL-6, and IL-1{beta}. J Biol Chem 285, 31185-31192.

Elferink, M.G., Olinga, P., Draaisma, A.L., Merema, M.T., Faber, K.N., Slooff, M.J., Meijer, D.K., Groothuis, G.M., 2004. LPS-induced downregulation of MRP2 and BSEP in human liver is due to a posttranscriptional process. American journal of physiology Gastrointestinal and liver physiology 287, G1008-1016.

Fardel, O., Le Vee, M., 2009. Regulation of human hepatic drug transporter expression by pro-inflammatory cytokines. Expert opinion on drug metabolism & toxicology 5, 1469-1481.

Fredriksson, L., Wink, S., Herpers, B., Benedetti, G., Hadi, M., de Bont, H., Groothuis, G., Luijten, M., Danen, E., de Graauw, M., Meerman, J., van de Water, B., 2014. Drug-induced endoplasmic reticulum and oxidative stress responses independently sensitize toward TNFalpha-mediated hepatotoxicity. Toxicological sciences : an official journal of the Society of Toxicology 140, 144-159.

Galle, P.R., Theilmann, L., Raedsch, R., Otto, G., Stiehl, A., 1990. Ursodeoxycholate reduces hepatotoxicity of bile salts in primary human hepatocytes. Hepatology 12, 486-491.

Ganey, P.E., Luyendyk, J.P., Maddox, J.F., Roth, R.A., 2004. Adverse hepatic drug reactions: inflammatory episodes as consequence and contributor. Chemico-biological interactions 150, 35-51.

Guillouzo, A., Guguen-Guillouzo, C., 2018. HepaRG Cells as a Model for Hepatotoxicity Studies. In: (Ed.), T.P.R. (Ed.) In Stem cells in toxicology and teratology, pp. 309-339. Hoboken, NJ, USA John Wiley & Sons, Inc.

Hao, H., Cao, L., Jiang, C., Che, Y., Zhang, S., Takahashi, S., Wang, G., Gonzalez, F.J., 2017. Farnesoid X Receptor Regulation of the NLRP3 Inflammasome Underlies Cholestasis-Associated Sepsis. Cell metabolism 25, 856-867 e855.

Klein, M., Thomas, M., Hofmann, U., Seehofer, D., Damm, G., Zanger, U.M., 2015. A systematic comparison of the impact of inflammatory signaling on absorption,

distribution, metabolism, and excretion gene expression and activity in primary human hepatocytes and HepaRG cells. Drug Metab Dispos 43, 273-283.

Larrey, D., Funck-Brentano, C., Breil, P., Vitaux, J., Theodore, C., Babany, G., Pessayre, D., 1983. Effects of erythromycin on hepatic drug-metabolizing enzymes in humans. Biochem Pharmacol 32, 1063-1068.

Le Vee, M., Lecureur, V., Stieger, B., Fardel, O., 2009. Regulation of drug transporter expression in human hepatocytes exposed to the proinflammatory cytokines tumor necrosis factor-alpha or interleukin-6. Drug metabolism and disposition: the biological fate of chemicals 37, 685-693.

Levine, C., Trivedi, A., Thung, S.N., Perumalswami, P.V., 2014. Severe ductopenia and cholestasis from levofloxacin drug-induced liver injury: a case report and review. Seminars in liver disease 34, 246-251.

Maiuri, A.R., Breier, A.B., Gora, L.F., Parkins, R.V., Ganey, P.E., Roth, R.A., 2015. Cytotoxic Synergy Between Cytokines and NSAIDs Associated With Idiosyncratic Hepatotoxicity Is Driven by Mitogen-Activated Protein Kinases. Toxicol Sci 146, 265-280.

Pomponio, G., Savary, C.C., Parmentier, C., Bois, F., Guillouzo, A., Romanelli, L., Richert, L., Di Consiglio, E., Testai, E., 2015. In vitro kinetics of amiodarone and its major metabolite in two human liver cell models after acute and repeated treatments. Toxicology in vitro : an international journal published in association with BIBRA 30, 36-51.

Roth, R.A., Maiuri, A.R., Ganey, P.E., 2017. Idiosyncratic Drug-Induced Liver Injury: Is Drug-Cytokine Interaction the Linchpin? The Journal of pharmacology and experimental therapeutics 360, 461-470.

Rubin, K., Janefeldt, A., Andersson, L., Berke, Z., Grime, K., Andersson, T.B., 2015. HepaRG cells as human-relevant in vitro model to study the effects of inflammatory stimuli on cytochrome P450 isoenzymes. Drug Metab Dispos 43, 119-125.

Russmann, S., Kaye, J.A., Jick, S.S., Jick, H., 2005. Risk of cholestatic liver disease associated with flucloxacillin and flucloxacillin prescribing habits in the UK: cohort study using data from the UK General Practice Research Database. Br J Clin Pharmacol 60, 76-82.

Sharanek, A., Burban, A., Burbank, M., Le Guevel, R., Li, R., Guillouzo, A., Guguen-Guillouzo, C., 2016. Rho-kinase/myosin light chain kinase pathway plays a key role in the impairment of bile canaliculi dynamics induced by cholestatic drugs. Scientific reports 6, 24709.

Sharanek, A., Burban, A., Humbert, L., Bachour-El Azzi, P., Felix-Gomes, N., Rainteau, D., Guillouzo, A., 2015. Cellular Accumulation and Toxic Effects of Bile Acids in Cyclosporine A-Treated HepaRG Hepatocytes. Toxicological sciences : an official journal of the Society of Toxicology 147, 573-587.

Sharanek, A., Burban, A., Humbert, L., Guguen-Guillouzo, C., Rainteau, D., Guillouzo, A., 2017. Progressive and Preferential Cellular Accumulation of

Hydrophobic Bile Acids Induced by Cholestatic Drugs Is Associated with Inhibition of Their Amidation and Sulfation. Drug metabolism and disposition: the biological fate of chemicals 45, 1292-1303.

Shaw, P.J., Ganey, P.E., Roth, R.A., 2010. Idiosyncratic drug-induced liver injury and the role of inflammatory stress with an emphasis on an animal model of trovafloxacin hepatotoxicity. Toxicological sciences : an official journal of the Society of Toxicology 118, 7-18.

Woolbright, B.L., Jaeschke, H., 2012. Novel insight into mechanisms of cholestatic liver injury. World journal of gastroenterology 18, 4985-4993.

Woolbright, B.L., Jaeschke, H., 2016. Therapeutic targets for cholestatic liver injury. Expert opinion on therapeutic targets 20, 463-475.

Yamazaki, H., Urano, T., Hiroki, S., Shimada, T., 1996. Effects of erythromycin and roxithromycin on oxidation of testosterone and nifedipine catalyzed by CYP3A4 in human liver microsomes. The Journal of toxicological sciences 21, 215-226.

Legends to figures

Figure 1. Effects of cytokines on caspase-3 activity and C-reactive protein release in HepaRG cell cultures exposed to individual or mixed pro-inflammatory cytokines.

HepaRG cell cultures were incubated with TNF-α (10 ng/ml), IL-6 (5 ng/ml) and IL-1β (0.5 ng/ml) individually or in mixture for 24 h. **(A)** Cytotoxicity was estimated using the caspase-3 activity assay. **(B)** CRP secretion levels were measured in the medium using ELISA assay. Caspase-3 activity is expressed as fold change compared to unexposed cultures arbitrarily set at a value of 1 and CRP as ng/ml. Data represent the means ± SEM of 3 independent experiments. *$p < 0.05$ compared to unexposed cells; $^{\$}p < 0.5$ cytokine mixture compared to individual cytokines.

Figure 2. Cytotoxic effects of antibiotics and pro-inflammatory cytokines in HepaRG cells.

(A-H) HepaRG cell cultures were incubated with different concentrations of FLX **(A)**, CLX **(B),** NAF **(C)** TVX **(D),** LVX **(E),** ERY **(F)**, SM **(G)** and AMP **(H)** in the absence or presence of cytokines. Cells were first incubated with cytokines for 24 h before simultaneous incubation with ATBs for another 24 h. Cytokines were used either individually or in mixture at the following concentrations: TNF-α (10 ng/ml), IL-6 (5 ng/ml) and IL-1β (0.5 ng/ml). Cytotoxicity was measured using the caspase-3 activity assay. Data were expressed relative to those obtained with untreated cells arbitrarily set at 1. They represent the means ± SEM of 3 independent experiments. *$p < 0.05$ compared to untreated cells; $^{\#}p < 0.05$ ATB + cytokines individually compared to ATB alone; $^{\$}p < 0.05$ ATB + cytokine mixture compared to ATB alone and ATB + individual cytokine.

Figure 3. Influence of antibiotics and pro-inflammatory cytokines on bile canaliculi structures of HepaRG cells.

(A) Representative phase-contrast images showing BC dilatation (arrows) in HepaRG cells treated for 2 h with non-cytotoxic ATB concentrations in absence or presence of individual and combined pro-inflammatory cytokines. Cells were first incubated with cytokines for 24 h before simultaneous incubation with ATBs. Phase-contrast images were captured using time-lapse microscopy (bar = 50 µm).

(**B-D**) Mean bile canaliculi (BC) areas of HepaRG hepatocytes treated with ATBs for 2 h in absence or presence of individual or combined cytokines or exposed to cytokines alone, using ImageJ 1.48 software as described in the Materials and Methods section. Data were expressed as the fold change in the BC mean area relative to the mean area of untreated cells arbitrarily set at a value of 1. They represent the means ± SEM of 3 independent experiments. *$p < 0.05$ compared to untreated cells; $^{\#}p < 0.05$ ATB + individual cytokines compared to ATB alone. (A-D): ATBs were used at the following concentrations: FLX, CLX and NAF: 2 mM; TVX: 20 µM; LVX: 2 mM; ERY: 100 µM AMP: 24 mM and SM: 15mM.

Figure 4. [^{3}H]-TCA uptake and efflux in HepaRG cells treated with antibiotics and pro-inflammatory cytokines separately or in combination

(A) [^{3}H]-TCA uptake in HepaRG cells after 24 h treatment with pro-inflammatory cytokines separately or in combination. **(B,C)** [^{3}H]- efflux in HepaRG cells treated with pro-inflammatory cytokines alone (**B**) or co-treated with pro-inflammatory cytokines and the different ATBs **(C)** for 2 h. Cytokine concentrations were the following: TNF-α (10 ng/ml), IL-6 (5 ng/ml) and IL-1β (0.5 ng/ml). The data were expressed relative to

that of untreated cells arbitrarily set at 100% and are presented as the means +/- SEM of 3 independent experiments. *p < 0.05 compared with that of the untreated cells.

Figure 5. Effects of antibiotics and pro-inflammatory cytokines on CDF efflux in HepaRG cells.

(A) Representative fluorescent images showing CDF canalicular efflux in HepaRG hepatocytes treated for 2 h with the different ATBs in absence or presence of the cytokine mixture. Cells were first incubated with cytokines for 24 h before simultaneous incubation with ATBs for 2 h. **(B-D)** Quantification of CDF accumulation in BC of HepaRG cells after 2 h treatment with ATBs in absence or presence of individual or combined cytokines or exposed to cytokines alone (D), using imageJ 1.48 software as described in the Materials and Methods section. Fluorescent images were obtained with a Cellomics ArrayScan VTI HCS Reader (bar = 50 µm). Hoechst-labelled nuclei in blue. Data were expressed relative to those of untreated cells arbitrarily set at 100%. They represent the means ± SEM of 3 independent experiments. *$p < 0.05$ compared to untreated cells; $^{\#}p < 0.05$ ATB + individual cytokines compared to ATB alone.

Highlights

- HepaRG cells were co-exposed to cholestatic antibiotics and pro-inflammatory cytokines.
- Bile canaliculi dilatation induced by cholestatic antibiotics was enhanced by IL-6 and IL-1β.
- Efflux inhibition of CDF, a MRP2 substrate, was aggravated in response to pro-inflammatory cytokines.
- Antibiotic-induced cholestatic changes were more sensitive than cytotoxicity to inflammatory stress.
- The extent of effects were dependent on the cytokine and the cholestatic drug.

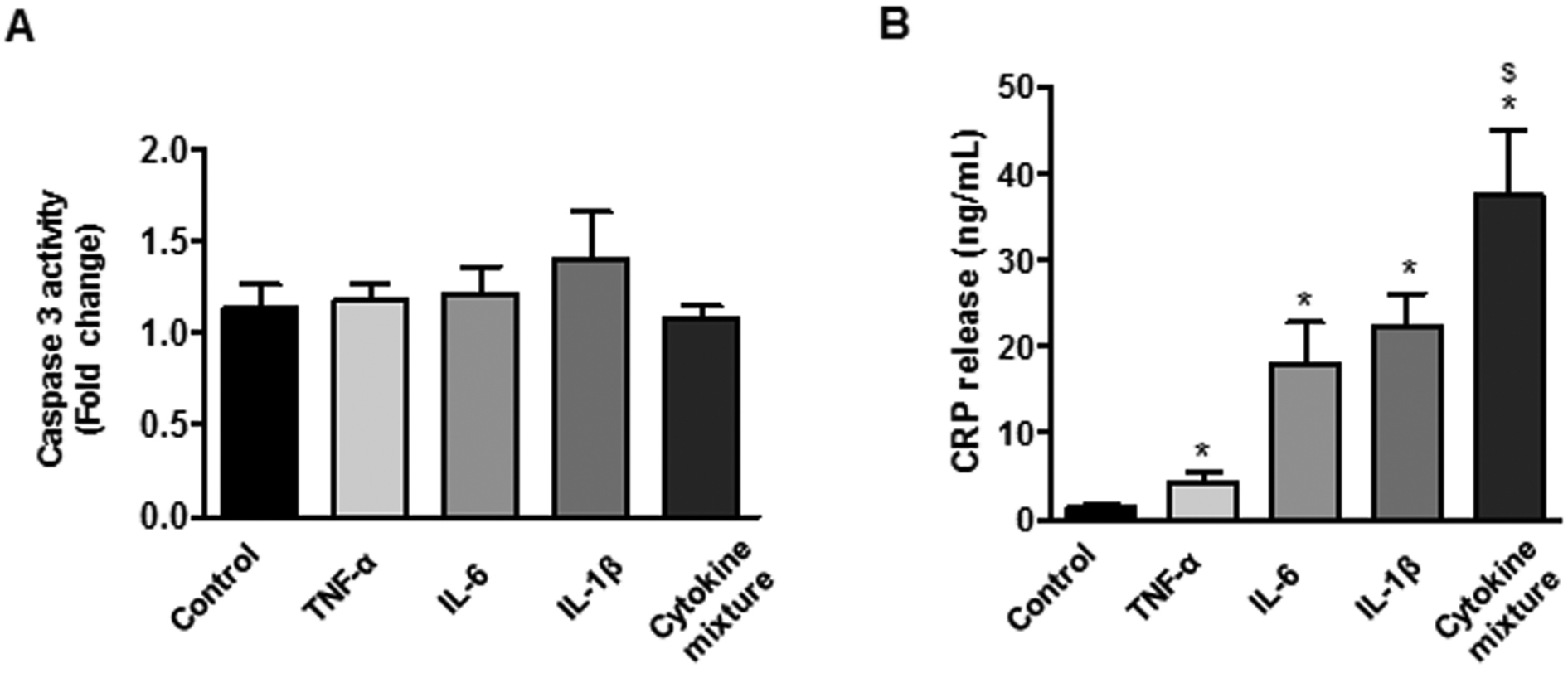
A
Caspase 3 activity
(Fold change)
2.0
1.5
1.0
0.5
0.0
Control
TNF-α
IL-6
IL-1β
Cytokine mixture
B
CRP release (ng/mL)
50
40
30
20
10
0
*
*
*
$
*
Control
TNF-α
IL-6
IL-1β
Cytokine mixture

Figure 1

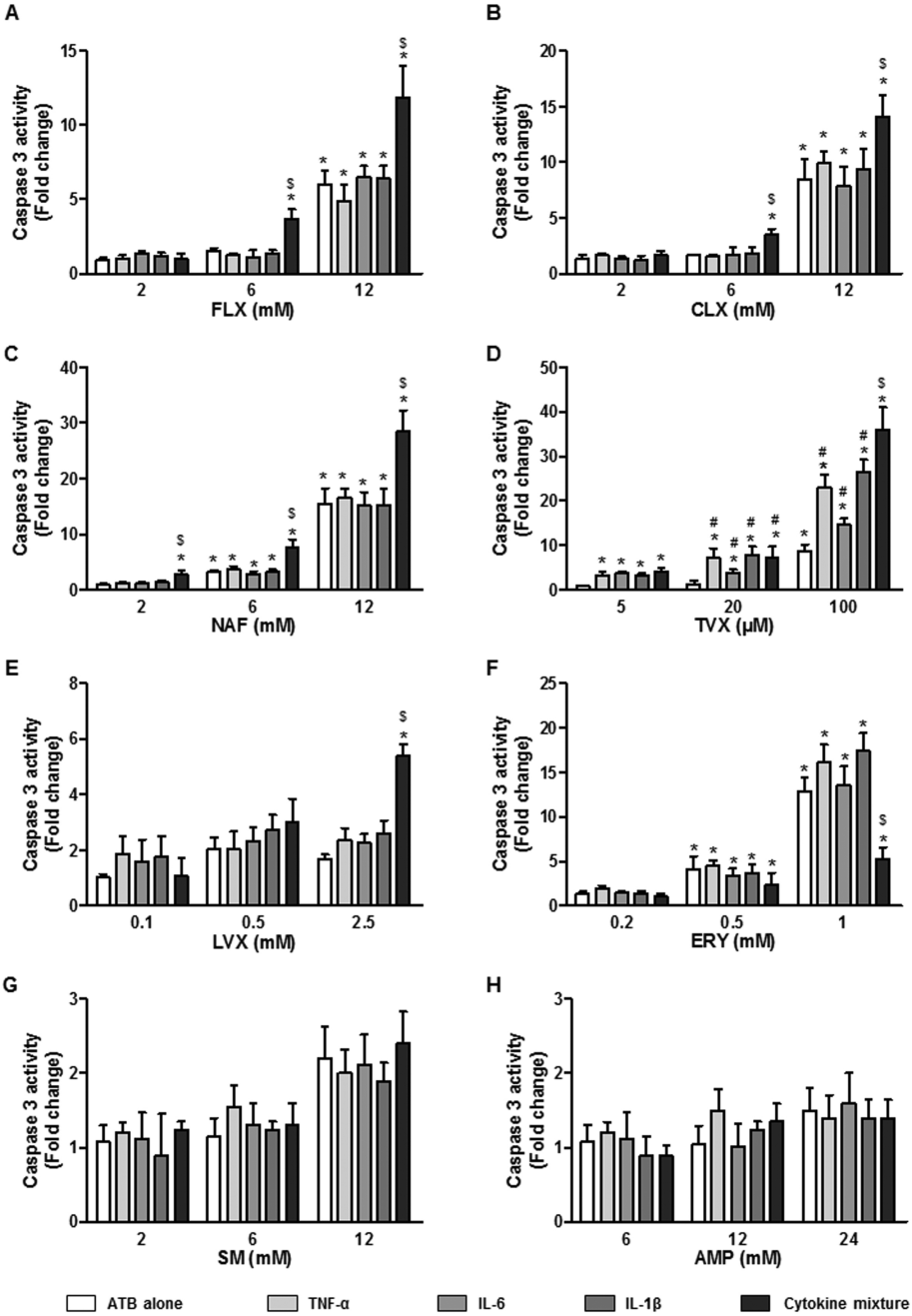
A
Caspase 3 activity (Fold change)
FLX (mM)
B
CLX (mM)
C
NAF (mM)
D
TVX (μM)
E
LVX (mM)
F
ERY (mM)
G
SM (mM)
H
AMP (mM)
ATB alone
TNF-α
IL-6
IL-1β
Cytokine mixture

Figure 2

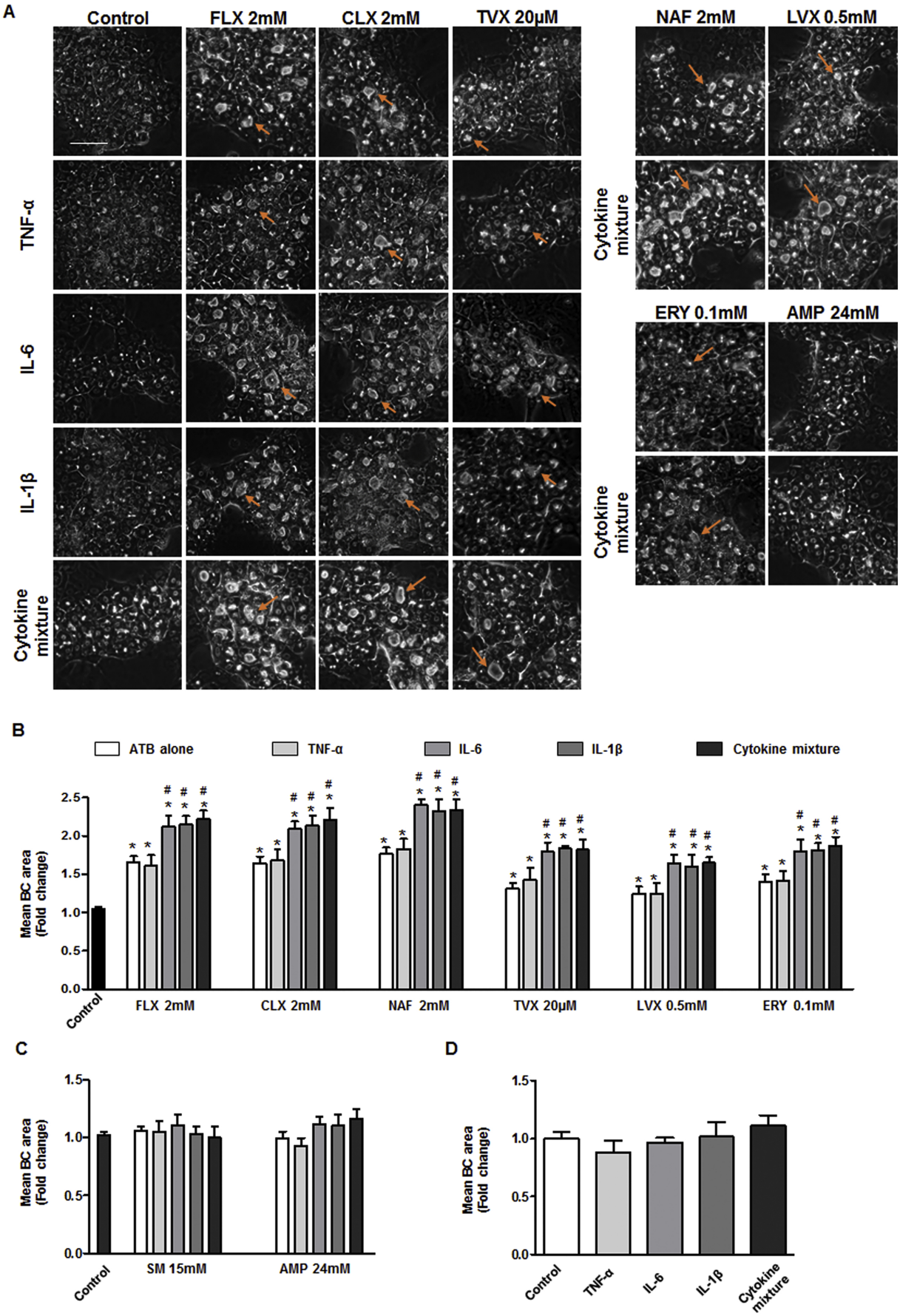
A
Control
FLX 2mM
CLX 2mM
TVX 20μM
NAF 2mM
LVX 0.5mM
TNF-α
IL-6
IL-1β
Cytokine mixture
Cytokine mixture
ERY 0.1mM
AMP 24mM
Cytokine mixture
B
ATB alone
TNF-α
IL-6
IL-1β
Cytokine mixture
Mean BC area (Fold change)
2.5
2.0
1.5
1.0
0.5
0.0
Control
FLX 2mM
CLX 2mM
NAF 2mM
TVX 20μM
LVX 0.5mM
ERY 0.1mM
C
Mean BC area (Fold change)
1.5
1.0
0.5
0.0
Control
SM 15mM
AMP 24mM
D
Mean BC area (Fold change)
1.5
1.0
0.5
0.0
Control
TNF-α
IL-6
IL-1β
Cytokine mixture

Figure 3

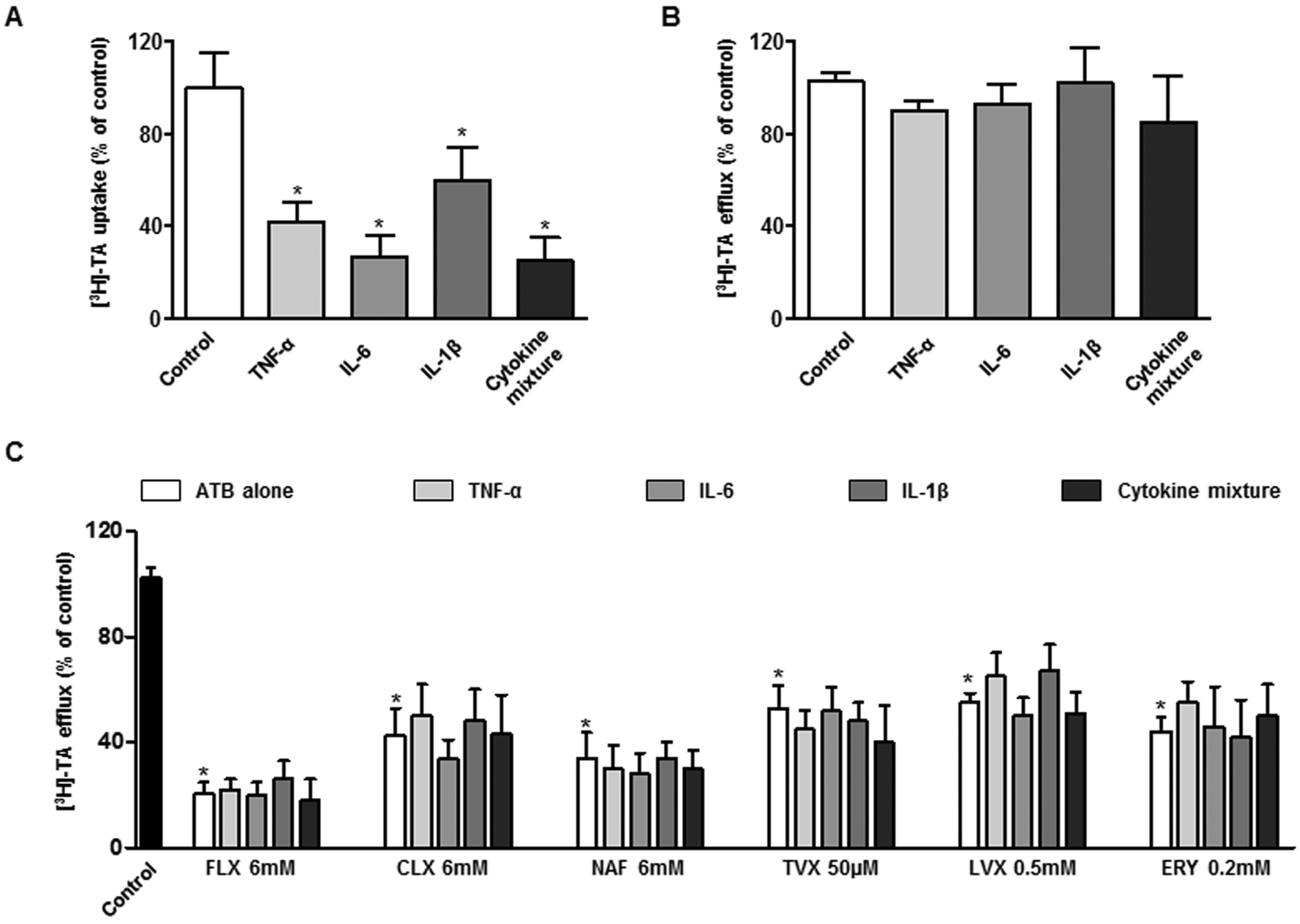
A
[3H]-TA uptake (% of control)
120
80
40
0
Control
TNF-α
IL-6
IL-1β
Cytokine mixture
B
[3H]-TA efflux (% of control)
120
80
40
0
Control
TNF-α
IL-6
IL-1β
Cytokine mixture
C
ATB alone
TNF-α
IL-6
IL-1β
Cytokine mixture
[3H]-TA efflux (% of control)
120
80
40
0
Control
FLX 6mM
CLX 6mM
NAF 6mM
TVX 50μM
LVX 0.5mM
ERY 0.2mM

Figure 4

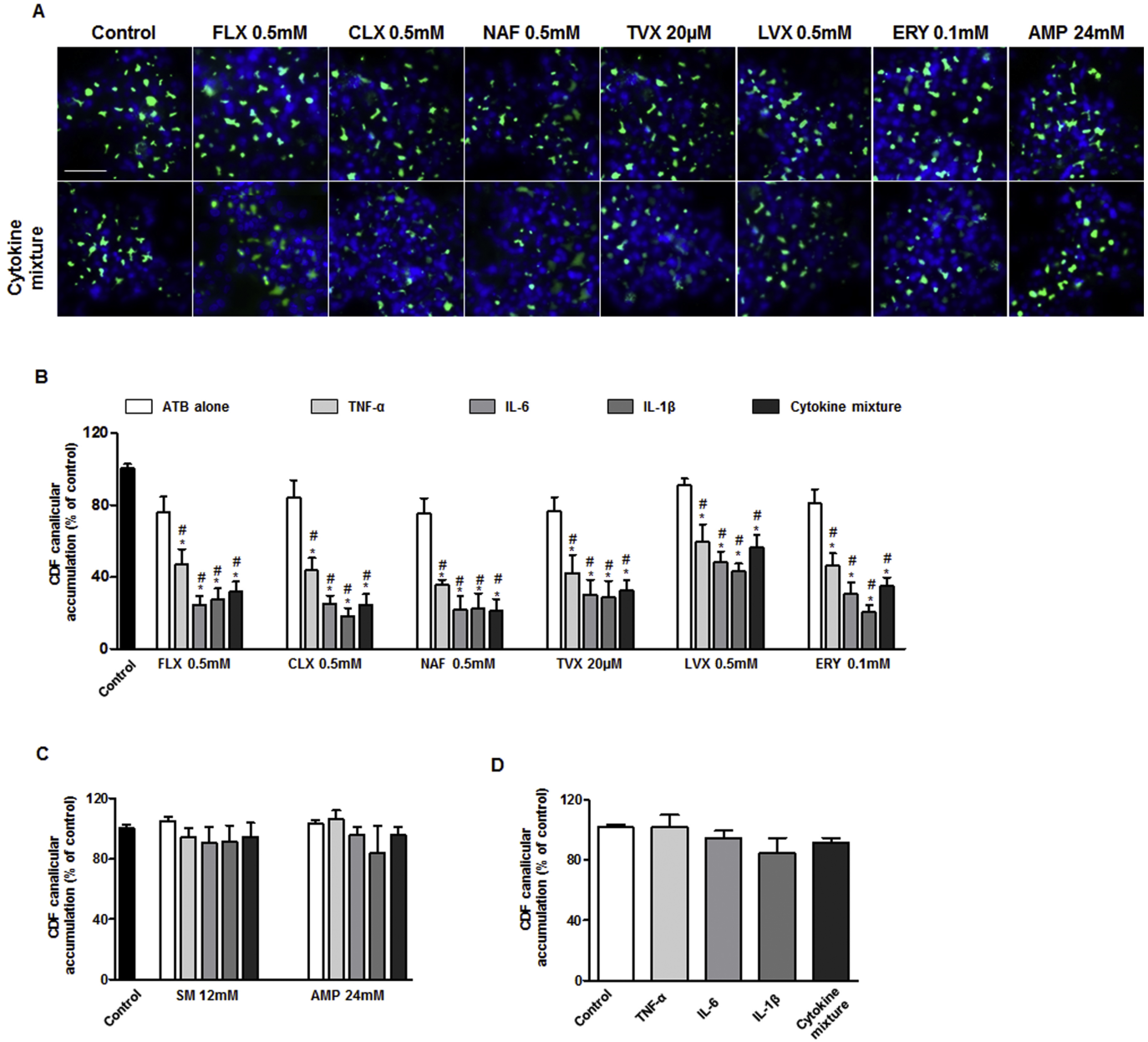
A
Control
FLX 0.5mM
CLX 0.5mM
NAF 0.5mM
TVX 20μM
LVX 0.5mM
ERY 0.1mM
AMP 24mM
Cytokine mixture
B
ATB alone
TNF-α
IL-6
IL-1β
Cytokine mixture
CDF canalicular accumulation (% of control)
120
80
40
0
Control
FLX 0.5mM
CLX 0.5mM
NAF 0.5mM
TVX 20μM
LVX 0.5mM
ERY 0.1mM
C
CDF canalicular accumulation (% of control)
120
80
40
0
Control
SM 12mM
AMP 24mM
D
CDF canalicular accumulation (% of control)
120
80
40
0
Control
TNF-α
IL-6
IL-1β
Cytokine mixture

Figure 5