Original Research

Co-incubation of PMN and CaCo-2 cells modulates inflammatory potential

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Abstract: Polymorphonuclear granulocytes (PMN) are activated in inflammatory reactions. Intestinal epithelial cells are relevant for maintaining the intestinal barrier. We examined interactions of PMN and intestinal epithelial cell-like CaCo-2 cells to elucidate their regulation of inflammatory signalling and the impact of cyclooxygenase (COX), nitric oxide (NO) and platelet-activating factor (PAF). Human PMN and CaCo-2 cells, separately and in co-incubation, were stimulated with the calcium ionophore A23187 or with N-Formyl-methionyl-leucyl-phenylalanin (fMLP) that activates PMN only. Human neutrophil elastase (HNE) and respiratory Burst were measured. To evaluate the modulation of inflammatory crosstalk we applied inhibitors of COX (acetyl salicylic acid; ASA), NO-synthase (N-monomethyl-L-arginin; L-NMMA), and the PAF-receptor (WEB2086). Unstimulated, co-incubation of CaCo-2 cells and PMN led to significantly reduced Burst and elevated HNE as compared to PMN. After stimulation with A23187, co-incubation resulted in an inhibition of Burst and HNE. Using fMLP co-incubation failed to modulate Burst but increased HNE. Without stimulation, all three inhibitors abolished the effect of co-incubation on Burst but did not change HNE. ASA partly prevented modulation of Burst. L-NMMA and WEB2086 did not change Burst but abolished mitigation of HNE. Without stimulation, co-incubation reduced Burst and elevated HNE. Activation of PMN and CaCo-2 cells by fMLP as compared to A23187 resulted in a completely different pattern of Burst and HNE, possibly due to single vs. dual cell activation. Anti-inflammatory effect of co-incubation might in part be due to due to COX-signalling governing Burst whereas NO- and PAF-dependent signalling seemed to control HNE release.

Key word: Polymorphonuclear granulocytes; CaCo-2 cells; Inflammation; Nitric oxide; Platelet-activating factor.

Introduction

The intestine plays a major role in inflammation not only orchestrating the mucosal immune response but also due to its epithelial barrier function (1). The co-operation of polymorphonuclear neutrophils (PMN) and intestinal epithelial cells in inflammatory signalling is an underestimated but important concept (2). Furthermore, PMN are the first line of defence against infection maintaining epithelial integrity.

The intestine makes up an enormous surface within the body being one of the most relevant barriers for invading pathogens; and its integrity is of tremendous importance for survival of the host (1). In inflammation, dysfunction between intestinal epithelial cells and PMN plays a major role and may lead to failure of the intestine barrier with the fatal consequences of septicaemia, secondary acute organ failure, and septic shock. In fact, the gut is recognized as the motor of sepsis with sepsis being the leading cause of death in non-coronary intensive care units (3, 4).

PMN modulate inflammation using a multitude of different mediators. Intercellular and paracrine signal transduction is governing their actions, e.g. via platelet activating factor (PAF) or nitric oxide (NO) (5-7). PAF is an inflammatory biolipid produced from membrane glycerophospholipids acting through the activity of its G-protein coupled receptor (8). NO can exert both protective and pro-inflammatory actions in the gastro-intestinal tract (9). Human neutrophil elastase (HNE) a serine protease released by PMN plays a key role in inflammatory diseases (10). It has been used as a marker of inflammation in CaCo-2 cells, a well-established intestinal epithelial cell line (2, 11). In PMN, HNE regulates other inflammatory and anti-inflammatory players like cytokines and chemokines (12). Reactive oxygen species like O^{2-}-anions promote inflammation by acting on the plasma membrane, damaging its organization and releasing various pro-inflammatory agents (13). Measuring O^{2-}-anions (respiratory Burst) is a recognized means to evaluate inflammation in PMN (14, 15) and in CaCo-2 cells (16, 17).

The aim of this study was to investigate PMN and CaCo-2 interaction in inflammation. Therefore, human PMN and CaCo-2 cells, separately and in co-incubation, were stimulated with the calcium ionophore A23187 or with N-Formyl-methionyl-leucyl-phenylalanin (fMLP). A23187 is a non-receptor operating stimulus that induces an influx of calcium into the cell which leads to various inflammatory cellular effects e.g. promotion of PAF signalling. The bacterial chemotactic peptide fMLP is a receptor operating inflammatory stimulus with its receptor found on PMN but not on CaCo-2 cells (18-21). To evaluate the modulation of inflammatory crosstalk

between the different cells we measured HNE and respiratory Burst and applied inhibitors of cyclooxygenase (COX: acetylsalicylic acid; ASA), NO-synthase (N-monomethyl-L-arginine; L-NMMA), and the PAF-receptor (WEB2086).

Materials and Methods

Materials

Chemicals of highest purity were obtained from Merck (Darmstadt, Gernany). fMLP, superoxide dismutase (SOD), cytochrome c and bovine serum albumin (BSA) were bought from Sigma Chemical (Deisenhofen, Germany). Acetylsalicylic acid (ASA) was ordered from Bayer (Leverkusen, Germany), WEB2086 from Boehringer Ingelheim (Ingelheim, Germany) and L-NMMA as well as A23187 by Calbiochem (Bad Soden, Germany). Tissue culture plastic supplies were purchased from Becton-Dickinson (Heidelberg, Germany). Cell culture reagents, buffer, and media were from PAN (Aidenbach, Germany).

Isolation of PMN

Peripheral venous blood samples from healthy volunteers that were matched for gender and age were drawn. EDTA-anticoagulated blood was centrifuged in a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient and erythrocytes were sedimented using polyvinyl alcohol (Merck-Schuchardt, Hohenbrunn, Germany). PMN were isolated as described (22). PMN purity was > 98% (Pappenheim staining) and viability was 92-100 % (trypan blue exclusion) throughout.

Oxidative Burst

Superoxide anion (O_2^-) generation was assessed as superoxide dismutase-inhibitable reduction of cytochrome C according to Cohen (23) as described (24).

PMN, CaCo-2 cells, or co-incubations of PMN with CaCo-2 cells were activated according to the experimental protocol for 15 min in Hank´s balanced salt solution (HHBSS++).

Duplicate reaction mixtures containing cytochrome in the presence or absence of superoxide dismutase were performed at 37°C. Incubations were terminated by incubation on ice for 5 min followed by centrifugation at 4 °C at 1.200×g. O_2^- release was quantified as relative extinction at 546 nm in an Uvicon Spectrophotometer and expressed as nmol/ml of O_2^-.

Elastase

Elastase release was taken as marker for PMN degranulation and measured according to standard procedures (25, 26).

For induction of elastase release, PMN, CaCo-2 cells or co-incubations of PMN with CaCo-2 cells were activated according to the experimental protocol for 15 min in HHBSS++. Incubations were terminated by incubation on ice for 5 min followed by centrifugation at 4 °C at 1.200×g. The cell-free supernatant was harvested and analyzed as described above for elastase activity expressed in U/l in an Uvicon Spectrophotometer.

Cell culture

CaCo-2 cells were generously donated from Prof. H. Daniel, who had obtained them from ATCC (HTB 37, passage 31). Cells were cultured and passaged in Dulbecco's Modified Eagle Medium as previously described (27). CaCo-2 cells were used for experiments a) after being confluent and b) when measurements of brush border enzyme alkaline phosphatase (AP) reached a plateau phase occurring usually at day 14 of culture.

Detection of fMLP-receptor

Using FACScan from Becton Dickinson (Mountain View, CA, USA), PMN and CaCo-2 were examined for fMLP receptors applying fMLP-FITC/F1314 from Molecular Probes (Eugene, OR, USA) as described (28).

Experimental protocol

Culture media was removed and CaCo-2 cells were washed twice followed by a two hour incubation time with HHBSS++ at 37°C with 5% CO2 in an incubator in 12-well plates. In experiments with inhibitors, 300µM ASA, 10µM L-NMMA, or 10µM WEB 2086 were applied during that time. Then, HHBSS++ was removed. For measuring respiratory Burst, cytochrome or cytochrome/SOD solution was added. For measuring elastase, no cytochrome was added. In experiments with inhibitors, these were again applied. 5 million PMN per well were given to those sets of experiments running with PMN. A 15 min incubation time at 37°C in a water bath either with 0, 0.1, 1, or 10µM fMLP or 0, 0.1, 1, or 10 µM A23187 ensued.

Statistics

Data are given as mean ± SEM. Two-way analysis of variance and Student-Newman-Keuls post-hoc test was performed to test for differences between different experimental groups. Probability (p) values < 0.05 were considered to indicate statistical significance. Analysis was carried out using SigmaStat® version 3.5.

Results

Co-incubation of PMN and CaCo-2 inhibited respiratory Burst under control conditions and after stimulation with A23187.

CaCo-2 cells, PMN, as well as both cells in co-incubation were stimulated with 0, 0.1, 1, and 10 µM A23187. At any concentration, incubation CaCo-2 cells induced a respiratory Burst close to the detection limit. Under control conditions, co-incubation of PMN and CaCo-2 cells reduced respiratory Burst significantly compared to PMN (p<0.05; n=5-6; fig. 1a). Stimulation of PMN resulted in an increase to 0.133 ± 0.026 nmol/ml, 0.184 ± 0.024 nmol/ml, 0.254 ± 0.009 nmol/ml and 0.199 ± 0.021 nmol/ml, respectively. However, co-incubation of PMN and CaCo-2 cells led to a significant reduced respiratory Burst compared to PMN, with 0.052 ± 0.014 nmol/ml, 0.067 ± 0.015 nmol/ml, 0.090 ± 0.017 nmol/ml and 0.121 ± 0.020 nmol/ml, respectively (p<0.05; n=5-6; fig. 1a).

Co-incubation of PMN and CaCo-2 did not modify respiratory Burst after stimulation with fMLP.

CaCo-2 cells, PMN, as well as both cells in co-incu-

bation were stimulated with 0, 0.1, 1, and 10 µM fMLP. Under control conditions, co-incubation of PMN and CaCo-2 cells reduced respiratory Burst significantly, compared to PMN (p<0.05; n=7; fig. 1b). Stimulation of PMN led to a respiratory Burst of 0.122 ± 0.035 nmol/l, 0.188 ± 0.033 nmol/l, 0.221 ± 0.025 nmol/l and 0.205 ± 0.034 nmol/l, respectively; but stimulation of CaCo-2 cells induced a respiratory Burst close to the detection limit at any concentration. Co-incubation of PMN and CaCo-2 cells did not modify respiratory Burst significantly compared to PMN (n=7; fig. 1b).

ASA partly abolished reduction of respiratory Burst by co-incubation of PMN and CaCo-2 under control conditions and after stimulation with A23187.

Stimulation of PMN with 0, 0.1, 1, and 10 µM A23187 in the presence of ASA resulted in a respiratory Burst of 0.053 ± 0.013 nmol/ml, 0.138 ± 0.039 nmol/ml, 0.227 ± 0.053 nmol/ml and 0.196 ± 0.043 nmol/ml, respectively. Stimulation of CaCo-2 cells led to a minor respiratory Burst. When PMN and CaCo-2 cells were stimulated in co-incubation with 0.1 and 10 µM A23187 and under control conditions, ASA prevented a significant reduction of respiratory Burst (n=4-5; fig. 1c).

L-NMMA prevented a reduction of respiratory Burst by co-incubation of PMN and CaCo-2 under control conditions but did not influence the modulation after stimulation with A23187.

CaCo-2 cells, PMN, as well as both cells in co-incubation were stimulated in the presence of L-NMMA with A23187 as detailed. Stimulation of CaCo-2 cells led to a respiratory Burst close to the detection limit. Under control conditions, L-NMMA prevented the co-incubation induced reduction of Burst. L-NMMA did not change the pattern of reduced release of superoxide anions in the setting of co-incubation (p<0.05; n=4-5; fig. 1d).

WEB2086 prevented a reduction of respiratory Burst by co-incubation of PMN and CaCo-2 under control conditions but did not influence the modulation after stimulation with A23187.

CaCo-2 cells, PMN, as well as both cells in co-incubation were stimulated with A23187 in the presence of WEB2086 as detailed. Incubation of CaCo-2 cells resulted in a Burst close to the detection limit. Under control conditions, WEB2086 prevented a co-incubation induced reduction of Burst. The co-incubation dependent reduction of Burst was not modified by WEB2086
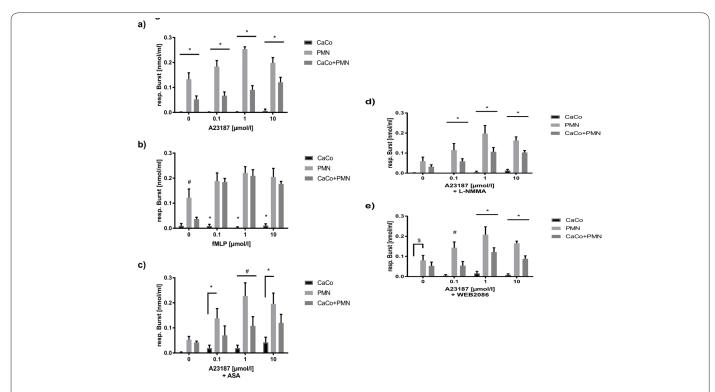

Figure 1. For analysis of respiratory Burst, CaCo-2 cells and PMN separately as well as in co-incubation were stimulated with 0, 0.1, 1, and 10 µM A23187 (a, c, d, e) or with 0, 0.1, 1, and 10 µM fMLP (b). **a)** CaCo-2 cells differed significantly from both other groups with PMN present (*, p<0.05; n=5-6). Co-incubation of PMN and CaCo-2 cells led to a significant reduced respiratory Burst compared to PMN stimulated separately (*, p<0.05; n=5-6). **b)** After stimulation with fMLP, CaCo-2 cells differed significantly from both other groups with PMN present (*, p<0.05; n=7). In control experiments, CaCo-2 cells were significantly different to the PMN group only (#, p<0.05 vs. PMN; n=7) but co-incubation with CaCo-2 cells led to a decrease compared to the PMN group. After stimulation with fMLP, co-incubation of PMN and CaCo-2 did not modify respiratory Burst significantly compared to PMN though in control experiments. **c)** In experiments applying the COX-inhibitor ASA, CaCo-2 cells differed significantly from the PMN group (*, p<0.05; n=4-5) but in the control experiments. Using 1 µM A23187, all groups differed significantly from each other (#, p<0.05, n=4-5). Stimulating with 0.1 and 10 µM A23187, ASA prevented a significant co-incubation-dependent reduction (n=4-5). **d)** In experiments applying the NO-inhibitor L-NMMA, all groups differed significantly from each other (*, p<0.05; n=4-5) after stimulation, while in the control group no significant differences were found. **e)** In experiments applying the PAF-inhibitor WEB2086, the PMN group differed significantly from the CaCo-2 group in all settings ($, #, *, p<0.05, n=4). Using 0.1 µM A23187, PMN cells were also significantly different from the co-incubation group (#, p<0.05, n=4). Applying 1 and 10 µM A23187, all groups differed significantly from each other (*, p<0.05, n=4). Data are given as mean ± SEM.

stimulating with 1 or 10 µM A23187 (p<0.05, n=4; fig. 1e).

Co-incubation of PMN and CaCo-2 increased elastase under control conditions but led to a reduction after stimulation with A23187.

Stimulation of CaCo-2 cells did not lead to a release of elastase at neither concentration of A23187. Measurement of elastase after stimulation of PMN with 0, 0.1, 1 and 10 µM A23187 revealed 1.7 ± 0.4 U/l, 3.7 ± 1.8 U/l, 9.5 ± 1.8 U/l and 12.7 ± 1.7 U/l, respectively. Under control conditions, co-incubation led to a significant increase of elastase (p<0.05; n=6; fig 2a). Interestingly, co-incubation of PMN and CaCo-2 cells resulted in a significant reduced elastase release when stimulated with 1 and 10 µM A23187 (p<0.05; n=6; fig 2a).

Co-incubation of PMN and CaCo-2 increased elastase release under control conditions and after stimulation with fMLP.

CaCo-2 cells, PMN, as well as both cells in co-incubation were stimulated with 0, 0.1, 1, and 10 µM fMLP. Stimulation of CaCo-2 cells did not induce an elastase release at neither concentration of fMLP. Stimulation of PMN led to an elastase release of 1.7 ± 0.4 U/l, 2.0 ± 0.3 U/l, 1.9 ± 0.3 U/l and 2.1 ± 0.3 U/l, respectively. However, without and with stimulation co-incubation of PMN and CaCo-2 cells resulted in a significantly in-

creased elastase release compared to PMN with 3.3 ± 0.6 U/l, 7.4 ± 1.5, 7.4 ± 1.2 and 7.6 ± 1.3, respectively (p<0.05; n=6; fig. 2b).

ASA failed to modulate elastase release by co-incubation of PMN and CaCo-2 under control conditions and after stimulation with A23187.

The cells were stimulated with A23187 in the presence of ASA as described. While stimulation of CaCo-2 cells resulted in an elastase release close to the detection limit, stimulation of PMN induced an elastase release of 1.4 ± 0.2 U/l, 3.8 ± 1.3 U/l, 11.8 ± 2.7 U/l and 18.2 ± 1.2 U/l, after stimulation with 0, 0.1, 1 and 10 µM A23187, respectively. Co-incubation of PMN and CaCo-2 cells with or without ASA resulted in a similar pattern of reduced elastase release (p<0.05; n=5; fig. 2c).

L-NMMA did not change pattern under control conditions but modulated the reduced elastase release by co-incubation of PMN and CaCo-2 after stimulation with A23187.

CaCo-2 cells, PMN as well as both cells in co-incubation were stimulated with A23187 in the presence of L-NMMA as detailed. Stimulation of CaCo-2 cells resulted in an elastase release close to the detection limit. L-NMMA did not modify the pattern of elastase release under control conditions. In contrast, when PMN and CaCo-2 cells were stimulated with 1 or 10 µM A23187,
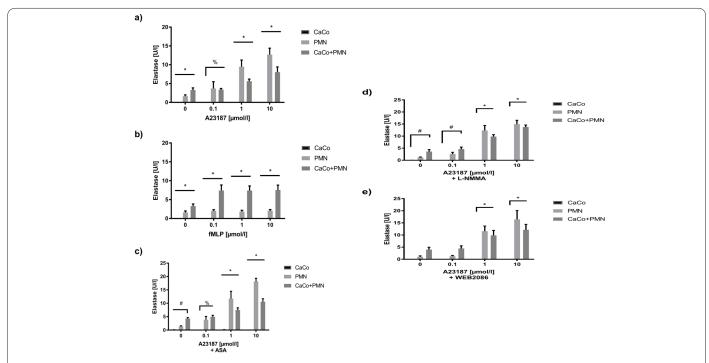

Figure 2. For analysis of elastase release, CaCo-2 cells and PMN separately as well as in co-incubation were stimulated with 0, 0.1, 1, and 10 µM A23187 (a, c, d, e) or with 0, 0.1, 1, and 10 µM fMLP (b). In all experiments CaCo-2 did yield elastase close to detection limits. **a)** CaCo-2 cells differed significantly from both other groups in all experimental settings (*, %, p<0.05; n=6). Under control conditions, co-incubation increased elastase significantly compared to PMN (*, p<0.05). 0.1 µM A23187 induced a significant difference only between CaCo-2 cells and both other groups (%, p<0.05). Co-incubation of PMN and CaCo-2 cells resulted in a significant reduced elastase release when stimulating with 1 and 10 µM A23187 (*, p<0.05). **b)** All groups differed significantly from each other with the co-incubation group inducing the highest elastase release (*, p<0.05; n=6-7). **c)** In all experiments applying the COX inhibitor ASA, CaCo-2 cells differed significantly from the co-incubation group (#, %, *, p<0.05; n=5); after stimulation with A23187 CaCo-2 differed also from the PMN group (%, *, p<0.05). Co-incubation reduced elastase compared to PMN significantly using 1 and 10 µM A23187 (*, p<0.05). **d)** In experiments applying the NO-inhibitor L-NMMA using 0 and 0.1 µM A23187, CaCo-2 cells differed significantly from CaCo-2 co-incubation group (#, p<0.05; n=5). At concentrations of 1 and 10 µM A23187, CaCo-2 cells differed significantly from both other groups (*, p<0.05; n=5). **e)** In experiments applying the PAF-inhibitor WEB2086, stimulating with 1 and 10 µM A23187, the CaCo-2 group differed significantly from the PMN and from co-incubation group (*, p<0.05; n=4). Data are given as mean ± SEM.

L-NMMA prevented the significant co-incubation-dependent inhibition of elastase production (p<0.05; n=5; fig. 2d).

WEB2086 did not change pattern of elastase release under control conditions but abolished the co-incubation-dependent reduction of elastase.

CaCo-2 cells, PMN, or both cells in co-incubation were stimulated with A23187 in the presence of WEB2086 as detailed. Incubating CaCo-2 cells resulted in a non-detectable elastase release. WEB2086 did not modify the pattern of elastase release under control conditions. Interestingly, WEB2086 prevented a co-incubation-dependent reduction of elastase release when stimulated with 1 or 10 µM A23187 (p<0.05; n=4; fig. 2e).

Discussion

The aim of this study was to investigate interaction of PMN and CaCo-2 under inflammatory conditions. Therefore, human PMN and CaCo-2 cells, separately and in co-incubation, were stimulated with the calcium ionophore A23187 or with fMLP. Under control conditions, co-incubation of PMN and CaCo-2 cells reduced Burst and elevated HNE as compared to PMN. When stimulated with A23187, co-incubation of CaCo-2 cells and PMN as compared to PMN alone reduced release of HNE and Burst significantly. This reduction was not found using fMLP. The co-incubation induced reduction of Burst seen under control conditions was not present after stimulation with fMLP. Instead, fMLP induced a similar Burst of PMN alone and in co-incubation.

Under control conditions and after stimulation with fMLP, HNE was increased in co-incubation experiments compared to PMN. FMLP did not induce a dose-dependent increase in HNE of PMN alone. Though, co-incubation resulted in an enhanced release of HNE displaying a ceiling-effect.

In control setting, all three inhibitors (ASA, L-NM-MA and WEB2086) abolished the inhibitory effect of co-incubation on Burst but did not change pattern of HNE. Interestingly, only after stimulation with A23187 all three inhibitors provoked a slightly stronger CaCo-2 response in Burst compared to experiments without inhibitors with ASA being the most effective one. This was not the case in HNE experiments. ASA partly influenced the co-incubation dependent reduction in respiratory Burst significantly but failed to modulate HNE. However, using inhibitors of the NO-synthase and PAF, Burst was unchanged but the mitigation of HNE release was abolished after stimulation with A23187.

The model of PMN and CaCo-2 co-incubation to evaluate inflammation is well recognized in literature (29). Using this model, we could demonstrate that under control conditions and after stimulation with A23187, co-incubation of PMN and CaCo-2 inhibited respiratory Burst. In contrast, fMLP abolished co-incubation induced suppression of Burst seen under control conditions, but did not provoke a difference between PMN and PMN and CaCo-2 after stimulation. It has been already observed that intestinal epithelial cells like CaCo-2 cells may exert bactericidal activity (30). Though, in this inflammatory setting, we could demonstrate for the first time that a co-incubation of PMN and CaCo-2 cells led to a significant diminished 0^{2-} and elastase production after stimulation with A23187. In contrast, a pro-inflammatory effect of supernatant from hypoxia-re-oxygenation challenged CaCo-2 cells on PMN was described (31). However, this model deprived both cells from their direct interaction. Our experimental setting does not allow to determine if the effects observed are due to direct cell-cell contact or due to indirect – e.g. paracrine – mediators. Further experimental settings, e.g. using transwells, would be necessary to address this question.

Wang et al. showed that calcium ionophore A23187 in similar concentrations as used in our study increased intracellular calcium in CaCo-2 cell and initiated apoptosis in a dose dependent manner (32). Since the effect of A23187 on PMN is well recognized (33) we conclude that using A23187, we definitively stimulated both cell types. In contrast to the A23187-induced effect, our experiments revealed that co-incubation of PMN and CaCo-2 did not modify the respiratory Burst after stimulation with fMLP. We speculate that this effect is due to CaCo-2 cells lacking a fMLP-receptor and therefore an inflammatory signalling after challenge with fMLP. Supporting this notion, we did not find an expression of fMLP-receptor on the surface of our CaCo-2 cells using FACS. In contrast to our findings, Carlson et al. found that the polarized human intestinal epithelial cell line Caco2bbe could be stimulated with fMLP and this effect could be prevented using a fMLP-receptor antagonist (21). The difference in the two cells lines polarized versus non-polarized may be fully responsible inducing the contrasting results. In consequence, stimulating with fMLP actually led to a stimulation of PMN only. We assume this to be the reason why the co-operative inhibitory effect was lost when both cell types were stimulated using the receptor-independent stimulus A23187. In addition, Foster et al. pointed out that fMLP is absorbed by the intestinal oligopeptide transporter, hPEPT1, and is transported across CaCo-2 cells (34). This might also lead to a clearance of fMLP, but also to activation of alternative signalling pathways in CaCo-2 cells.

Considering the effect of co-incubation dependent inhibition of Burst we observed under control condition without any stimulation, we speculate that it must be due to a basal stimulation of both cell types e.g. by calcium. As a calcium ionophore, A23187 would increase this effect as demonstrated in our experiments. Though, fMLP would abolish the basal calcium-induced effect by its strong receptor-dependent activation of PMN only. This would explain, why we saw a basal co-incubation dependent inhibition of Burst that is abolished by fMLP.

We observed that co-incubation of PMN and CaCo-2 increased elastase release under control conditions and after stimulation with fMLP. Since elastase facilitates PMN transmigration (35), a concordant elevation when CaCo-2 meet PMN and with the chemoattractant fMLP appears to be a physiological reaction. In contrast, a previous study from another group showed a reduced elastase release after PMN and CaCo-2 co-incubation after hypoxia/re-oxygenation and bacterial challenge (11). The question remains why stimulation with A23187 induced a co-incubation dependent inhibition of elastase in contrast to basal conditions and to stimulation with fMLP. The contrasting effects of fMLP

and A23187 could be explained by the single vs. dual cell activation, respectively as explained above. The difference under basal conditions of elastase and Burst remain a subject for further investigations. One hypothesis could be that basal dual cell activation by calcium is not that effective in elastase experiments.

We speculate that the co-incubation dependent inhibition of Burst and HNE might depend on signalling pathways activated by A23187- induced calcium like those of cyclooxygenase, NO or PAF (36-38). We observed that inhibitors against cyclooxygenase (ASA), NO (L-NMMA) or PAF (WEB2086) all abolished the co-incubation induced inhibitory effect under basal conditions in Burst experiments. This seemed to be basically due to a slightly diminished PMN response when applying the inhibitors.

Analysing elastase, inhibitors did not change the pattern of increased elastase after co-incubation.

Our data depicted that ASA, an inhibitor of the cyclooxygenase, partly abolished the reduction of respiratory Burst but failed to modulate reduction of elastase release. We conclude that co-incubation dependent inhibition of Burst is in part related to cyclooxygenase signal transduction. Fukui et al. found that ASA-induced ROS (reactive oxygen species) production specifically modified the expression of ZO-1 protein and induced increased cell permeability, which may ultimately cause small intestinal mucosal injury (39). However, the concentrations ASA used (10mmol/l (39) vs. 300µmol/l) are not comparable. Nevertheless, a small effect of ASA on the ROS production may counteract the inhibitory effect due to the co-incubation. In line with our results, Egger et al. did not observe a significant alteration in PMN elastase detected in the blood of atherosclerosis patients after ASA treatment (40). We conclude that inhibiting cyclooxygenase in our setting has no significant effect on elastase release but slightly antagonized the reduction of O^{2-} production by PMN and CaCo-2 co-incubation. We speculate this might be due to ASA- induced ROS production in CaCo-2 cells (39).

NO is an important final effector of mucosal injury in inflammatory bowel disease (41). Therefore, we examined the effect of L-NMMA, an inhibitor of the nitric oxide synthase, on PMN and CaCo-2 co-incubation in inflammation. L-NMMA failed to significantly modulate reduction of respiratory Burst by co-incubation of PMN and CaCo-2 after stimulation with A23187. Though, our data indicated that L-NMMA did significantly modulate reduced elastase release under these conditions. We conclude that co-incubation-dependent inhibition of elastase after stimulation with A23187 is in part related to NO-signal transduction.

Brasse-Lagnel et al. demonstrated that the inhibitory effect of IL1-1β on argininosuccinate synthase activity is linked to the production of NO since it was totally blocked in the presence of L-NMMA. They resumed that such an inhibitory effect of NO may be related, at least in part, to S-nitrosylation of the proteins in CaCo-2 cells (42). Banan et al. proved that ethanol-induced microtubule skeleton damage and intestinal barrier dysfunction require iNOS activation followed by NO overproduction and ONOO⁻ formation (43). We do not think that iNOS does play a role in our setting as its induction required a longer term challenge with IFN-γ-containing

combinations of cytokines in CaCo-2 cells (44). However, NO attenuated elastase release from PAF-primed PMN through an intracellular cGMP-dependent signal transduction pathway (45). We think that NO-release is an important contributor to the reduced elastase release possibly by nitrosylation of (signalling)-proteins or by increasing intracellular cGMP.

We furthermore showed that the PAF-receptor inhibitor WEB2086 failed to significantly modulate the reduction of respiratory Burst by co-incubation of PMN and CaCo-2 after stimulation with A23187 but abolished the reduction of elastase release under these conditions. We conclude that co-incubation dependent inhibition of elastase after stimulation with A23187 is in part dependent on PAF-signal transduction. PAF is an important paracrine and autocrine mediator in PMN and CaCo-2 cells and its receptor is constitutively expressed and regulated (46). PAF-induced NF-κB activation may lead to IL-8 production in intestinal epithelial cells (47, 48). PAF and LTB4 may prime human PMN for the concordant release of elastase, generation of O^{2-}, and CD11b up-regulation. Therefore, PAF produced by PMN could play a role as positive feedback loop for further inflammatory activation (49). PAF-PAF-receptor interactions are involved in the adhesion of PMN to endothelial cells; inhibition of the PAF-receptor may reduce PMN adhesion to endothelial cells possibly by interfering with the outside-in signalling leading to firm adhesion (50). In differentiated CaCo-2 cells, infection by *S. enteritidis* increased PAF levels and the enzymes of the remodelling pathway cytosolic phospholipase A_2, which catalyses the formation of the PAF precursor lyso-PAF, and lyso-PAF acetyltransferase in a Ca^{2+}-dependent pathway (51). We conclude that the PAF-PAF-receptor signal transduction seemed to play an important role in both, PMN (52) and CaCo-2 inflammatory signalling, in particular in their cooperation.

It is interesting that different inhibitors of NO-synthase and PAF influenced the inhibition of elastase after PMN and CaCo-2 co-incubation but not the reduction in O^{2-} production. Released elastase might be responsible at least in part for PMN transmigration towards an inflammation focus through the intestinal epithelial barrier. Therefore, a tightly regulated release linked to multiple signalling pathways may ensure their way through the epithelium. Serine proteases like elastase activate specifically pro-inflammatory cytokines, lead to the activation of different receptors and act as key regulators of cell signalling during inflammation (53).

On the other hand, an overproduction of O^{2-} might damage both cell types. Therefore a reduction in O^{2-} generation due to co-operative inhibition if both cells are in close contact may be a useful mechanism to avoid mutual damage.

Another reason why elastase release but not respiratory Burst was modulated by NO- and PAF-inhibitors might be related to the fact that ROS may be produced by both, PMN and CaCo-2 cells, whereas elastase is only generated by PMN. Nevertheless, an interaction between both cell types may influence the release of the enzyme (54). This finding requires further investigation.

Other investigators also considered the migration of PMN across the intestinal epithelium as a histopathological hallmark of many mucosal inflammatory dis-

eases (55). Therefore, it is of relevance to further investigate the interaction of PMN and intestinal epithelial cells in inflammation. This might help understand regulatory mechanisms in inflammatory signalling. In consequence, a better and more specific treatment for patients suffering from intestinal inflammatory disorders should be available in future.

Without stimulation, PMN and CaCo-2 co-incubation reduced respiratory Burst. This might be due to cell-cell based interaction or a basal stimulation of both cell types since it was abolished by fMLP which only stimulates PMN. When stimulated in co-incubation by the calcium ionophore A23187, which affects both cell types, Burst and elastase release was inhibited. Cyclooxygenase might be in part relevant for co-incubation dependent inhibition of Burst since ASA partly abolished this anti-inflammatory effect.

Co-incubation increased elastase release under basal conditions and after stimulation with fMLP. When PMN and CaCo-2 were co-incubated, A23187 provoked an inhibition of elastase. NO- and PAF-signalling seemed to play an important role in the inhibitory signalling measuring elastase under conditions of co-incubation since L-NMMA and WEB2086 abolished the anti-inflammatory effect.

These findings might be relevant for the interactive signalling between neutrophils and intestinal epithelial cells in inflammatory diseases.

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Authors´ contribution

MBS wrote the manuscript. CAS carried out the experiments. MH and REM helped analysing and interpreting the results. MW and WS supervised the study. KM conceived the study and developed the design. All authors participated in analysing and interpreting the results, read and approved the final manuscript.

References

1. Sanchez de Medina F, Romero-Calvo I, Mascaraque C, Martinez-Augustin O. Intestinal inflammation and mucosal barrier function. Inflammatory bowel diseases 2014; 20(12):2394-2404.

2. Parlesak A, Haller D, Brinz S, Baeuerlein A, Bode C. Modulation of cytokine release by differentiated CACO-2 cells in a compartmentalized coculture model with mononuclear leucocytes and nonpathogenic bacteria. Scandinavian journal of immunology 2004; 60(5):477-485.

3. Klingensmith NJ, Coopersmith CM. The Gut as the Motor of Multiple Organ Dysfunction in Critical Illness. Critical care clinics 2016; 32(2):203-212.

4. Marshall JC, Dellinger RP, Levy M. The Surviving Sepsis Campaign: a history and a perspective. Surgical infections 2010; 11(3):275-281.

5. Jeon SY, Kim EA, Ma YW, Kim JP, Jung TG, Hwang EG. Nitric oxide mediates platelet activating factor-induced microvascular leakage in rat airways. The Annals of otology, rhinology, and laryngology 2001; 110(1):83-86.

6. Hossain M, Qadri SM, Liu L. Inhibition of nitric oxide synthesis enhances leukocyte rolling and adhesion in human microvasculature. Journal of inflammation 2012; 9(1):28.

7. Bussolati B, Mariano F, Migliori M, Camussi G. Nitric oxide/platelet activating factor cross-talk in mesangial cells modulates the interaction with leukocytes. Kidney international 2002; 62(4):1322-1331.

8. Melnikova V, Bar-Eli M. Inflammation and melanoma growth and metastasis: the role of platelet-activating factor (PAF) and its receptor. Cancer metastasis reviews 2007; 26(3-4):359-371.

9. Whittle BJ. Nitric oxide--a mediator of inflammation or mucosal defence. European journal of gastroenterology & hepatology 1997; 9(11):1026-1032.

10. Carbone F, Mach F, Montecucco F. Update on the role of neutrophils in atherosclerotic plaque vulnerability. Current drug targets 2015; 16(4):321-333.

11. Diebel LN, Liberati DM, Taub JS, Diglio CA, Brown WJ. Intestinal epithelial cells modulate PMN activation and apoptosis following bacterial and hypoxic challenges. The Journal of trauma 2005; 58(6):1126-1133.

12. Kessenbrock K, Dau T, Jenne DE. Tailor-made inflammation: how neutrophil serine proteases modulate the inflammatory response. Journal of molecular medicine 2011; 89(1):23-28.

13. Kaminski KA, Bonda TA, Korecki J, Musial WJ. Oxidative stress and neutrophil activation--the two keystones of ischemia/reperfusion injury. International journal of cardiology 2002; 86(1):41-59.

14. Grailer JJ, Kalbitz M, Zetoune FS, Ward PA. Persistent neutrophil dysfunction and suppression of acute lung injury in mice following cecal ligation and puncture sepsis. Journal of innate immunity 2014; 6(5):695-705.

15. Lei Y, Wang K, Deng L, Chen Y, Nice EC, Huang C. Redox regulation of inflammation: old elements, a new story. Medicinal research reviews 2015; 35(2):306-340.

16. Doskocil I, Havlik J, Verlotta R, Tauchen J, Vesela L, Macakova K *et al*. In vitro immunomodulatory activity, cytotoxicity and chemistry of some central European polypores. Pharmaceutical biology 2016:1-8.

17. Kawahara T, Kuwano Y, Teshima-Kondo S, Takeya R, Sumimoto H, Kishi K *et al*. Role of nicotinamide adenine dinucleotide phosphate oxidase 1 in oxidative burst response to Toll-like receptor 5 signaling in large intestinal epithelial cells. Journal of immunology 2004; 172(5):3051-3058.

18. Rotondo S, Dell'Elba G, Krauze-Brzosko K, Manarini S, Martelli N, Pecce R *et al*. Licofelone, a dual lipoxygenase-cyclooxygenase inhibitor, downregulates polymorphonuclear leukocyte and platelet function. European journal of pharmacology 2002; 453(1):131-139.

19. Schaible AM, Traber H, Temml V, Noha SM, Filosa R, Peduto A *et al*. Potent inhibition of human 5-lipoxygenase and microsomal prostaglandin E(2) synthase-1 by the anti-carcinogenic and anti-inflammatory agent embelin. Biochemical pharmacology 2013; 86(4):476-486.

20. Guo M, Yuan SY, Frederich BJ, Sun C, Shen Q, McLean DL *et al*. Role of non-muscle myosin light chain kinase in neutrophil-mediated intestinal barrier dysfunction during thermal injury. Shock 2012; 38(4):436-443.

21. Carlson RM, Vavricka SR, Eloranta JJ, Musch MW, Arvans DL, Kles KA *et al*. fMLP induces Hsp27 expression, attenuates NF-kappaB activation, and confers intestinal epithelial cell protection. American journal of physiology Gastrointestinal and liver physiology 2007; 292(4):G1070-1078.

22. Mayer K, Fegbeutel C, Hattar K, Sibelius U, Kramer HJ, Heuer KU *et al*. Omega-3 vs. omega-6 lipid emulsions exert differential influence on neutrophils in septic shock patients: impact on plasma

fatty acids and lipid mediator generation. Intensive Care Med 2003; 29(9):1472-1481.

23. Cohen HJ, Chovaniec ME. Superoxide generation by digitonin-stimulated guinea pig granulocytes. A basis for a continuous assay for monitoring superoxide production and for the study of the activation of the generating system. The Journal of clinical investigation 1978; 61(4):1081-1087.

24. Grimminger F, Hattar K, Papavassilis C, Temmesfeld B, Csernok E, Gross WL et al. Neutrophil activation by anti-proteinase 3 antibodies in Wegener's granulomatosis: role of exogenous arachidonic acid and leukotriene B4 generation. The Journal of experimental medicine 1996; 184(4):1567-1572.

25. Henson PM. The immunologic release of constituents from neutrophil leukocytes. II. Mechanisms of release during phagocytosis, and adherence to nonphagocytosable surfaces. J Immunol 1971; 107(6):1547-1557.

26. Kramps JA, van Twisk C, van der Linden AC. L-Pyroglutamyl-L-prolyl-L-valine-p-nitroanilide, a highly specific substrate for granulocyte elastase. Scand J Clin Lab Invest 1983; 43(5):427-432.

27. Wenzel U, Gebert I, Weintraut H, Weber WM, Clauss W, Daniel H. Transport characteristics of differently charged cephalosporin antibiotics in oocytes expressing the cloned intestinal peptide transporter PepT1 and in human intestinal Caco-2 cells. The Journal of pharmacology and experimental therapeutics 1996; 277(2):831-839.

28. Allen CA, Broom MF, Chadwick VS. Flow cytometry analysis of the expression of neutrophil FMLP receptors. Journal of immunological methods 1992; 149(2):159-164.

29. Amin PB, Diebel LN, Liberati DM. The intestinal epithelial cell modulates the effect of alcohol on neutrophil inflammatory potential. The Journal of trauma 2007; 63(6):1223-1229.

30. Deitch EA, Haskel Y, Cruz N, Xu D, Kvietys PR. Caco-2 and IEC-18 intestinal epithelial cells exert bactericidal activity through an oxidant-dependent pathway. Shock 1995; 4(5):345-350.

31. Amin PB, Diebel LN, Liberati DM. Secretory immunoglobulin a blunts gut-mediated priming of neutrophils in vitro. The Journal of trauma 2008; 64(6):1437-1442.

32. Wang L, Li Q, Duan XL, Chang YZ. Effects of extracellular iron concentration on calcium absorption and relationship between Ca2+ and cell apoptosis in Caco-2 cells. World journal of gastroenterology 2005; 11(19):2916-2921.

33. Rose F, Hattar K, Gakisch S, Grimminger F, Olschewski H, Seeger W et al. Increased neutrophil mediator release in patients with pulmonary hypertension--suppression by inhaled iloprost. Thrombosis and haemostasis 2003; 90(6):1141-1149.

34. Foster DR, Zheng X. Cephalexin inhibits N-formylated peptide transport and intestinal hyperpermeability in Caco2 cells. Journal of pharmacy & pharmaceutical sciences : a publication of the Canadian Society for Pharmaceutical Sciences, Societe canadienne des sciences pharmaceutiques 2007; 10(3):299-310.

35. Zen K, Guo YL, Li LM, Bian Z, Zhang CY, Liu Y. Cleavage of the CD11b extracellular domain by the leukocyte serprocidins is critical for neutrophil detachment during chemotaxis. Blood 2011; 117(18):4885-4894.

36. Cuschieri J, Bulger E, Garcia I, Jelacic S, Maier RV. Calcium/calmodulin-dependent kinase II is required for platelet-activating factor priming. Shock 2005; 23(2):99-106.

37. Datta D, Khatri P, Banerjee C, Singh A, Meena R, Saha DR et al. Calcium and Superoxide-Mediated Pathways Converge to Induce Nitric Oxide-Dependent Apoptosis in Mycobacterium fortuitum-Infected Fish Macrophages. PloS one 2016; 11(1):e0146554.

38. Anosov AK, Miramedova AG. Calcium-Induced Stimulation of Cyclooxygenase in Rabbit Leukocytes and its Modulation by Ultraviolet Radiation. Russ J Immunol 1997; 2(3-4):211-216.

39. Fukui A, Naito Y, Handa O, Kugai M, Tsuji T, Yoriki H et al.

Acetyl salicylic acid induces damage to intestinal epithelial cells by oxidation-related modifications of ZO-1. American journal of physiology Gastrointestinal and liver physiology 2012; 303(8):G927-936.

40. Egger G, Burda A, Obernosterer A, Mitterhammer H, Kager G, Jurgens G et al. Blood polymorphonuclear leukocyte activation in atherosclerosis: effects of aspirin. Inflammation 2001; 25(2):129-135.

41. Kennedy M, Wilson L, Szabo C, Salzman AL. 5-aminosalicylic acid inhibits iNOS transcription in human intestinal epithelial cells. International journal of molecular medicine 1999; 4(4):437-443.

42. Brasse-Lagnel C, Lavoinne A, Fairand A, Vavasseur K, Deniel N, Husson A. Biphasic effect of IL-1beta on the activity of argininosuccinate synthetase in Caco-2 cells. Involvement of nitric oxide production. Biochimie 2006; 88(6):607-612.

43. Banan A, Fields JZ, Decker H, Zhang Y, Keshavarzian A. Nitric oxide and its metabolites mediate ethanol-induced microtubule disruption and intestinal barrier dysfunction. The Journal of pharmacology and experimental therapeutics 2000; 294(3):997-1008.

44. Chavez AM, Menconi MJ, Hodin RA, Fink MP. Cytokine-induced intestinal epithelial hyperpermeability: role of nitric oxide. Critical care medicine 1999; 27(10):2246-2251.

45. Partrick DA, Moore EE, Offner PJ, Barnett CC, Barkin M, Silliman CC. Nitric oxide attenuates platelet-activating factor priming for elastase release in human neutrophils via a cyclic guanosine monophosphate-dependent pathway. Surgery 1997; 122(2):196-202; discussion 202-193.

46. Merendino N, Dwinell MB, Varki N, Eckmann L, Kagnoff MF. Human intestinal epithelial cells express receptors for platelet-activating factor. The American journal of physiology 1999; 277(4 Pt 1):G810-818.

47. Borthakur A, Bhattacharyya S, Kumar A, Anbazhagan AN, Tobacman JK, Dudeja PK. Lactobacillus acidophilus alleviates platelet-activating factor-induced inflammatory responses in human intestinal epithelial cells. PloS one 2013; 8(10):e75664.

48. Borthakur A, Bhattacharyya S, Alrefai WA, Tobacman JK, Ramaswamy K, Dudeja PK. Platelet-activating factor-induced NF-kappaB activation and IL-8 production in intestinal epithelial cells are Bcl10-dependent. Inflammatory bowel diseases 2010; 16(4):593-603.

49. Partrick DA, Moore EE, Moore FA, Barnett CC, Silliman CC. Lipid mediators up-regulate CD11b and prime for concordant superoxide and elastase release in human neutrophils. The Journal of trauma 1997; 43(2):297-302; discussion 302-293.

50. Zimmerman GA, McIntyre TM, Prescott SM. Adhesion and signaling in vascular cell-cell interactions. J Clin Invest 1997; 100(11 Suppl):S3-5.

51. Egea L, Gimenez R, Lucia D, Modolell I, Badia J, Baldoma L et al. Increased production of the ether-lipid platelet-activating factor in intestinal epithelial cells infected by Salmonella enteritidis. Biochimica et biophysica acta 2008; 1781(5):270-276.

52. Takahashi S, Yoshikawa T, Naito Y, Tanigawa T, Yoshida N, Kondo M. Role of platelet-activating factor (PAF) in superoxide production by human polymorphonuclear leukocytes. Lipids 1991; 26(12):1227-1230.

53. Wiedow O, Meyer-Hoffert U. Neutrophil serine proteases: potential key regulators of cell signalling during inflammation. Journal of internal medicine 2005; 257(4):319-328.

54. Bjornland K, Buo L, Scott H, Konttinen Y, Johansen HT, Aasen AO. Polymorphonuclear elastase in human colorectal carcinoma. International journal of oncology 1998; 12(3):535-540.

55. Brazil JC, Lee WY, Kolegraff KN, Nusrat A, Parkos CA, Louis NA. Neutrophil migration across intestinal epithelium: evidence for a role of CD44 in regulating detachment of migrating cells from the luminal surface. Journal of immunology 2010; 185(11):7026-7036.