Veterinary Immunology and Immunopathology 153 (2013) 45–56

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Escherichia coli induces bovine neutrophil cell death independent from caspase-3/-7/-1, but with phosphatidylserine exposure prior to membrane rupture

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ARTICLE INFO

Article history:
Received 8 February 2012
Received in revised form 11 January 2013
Accepted 4 February 2013

Keywords:
Bovine neutrophil
Phagocytosis
Escherichia coli
Cell death
Apoptosis
NETosis
Necroptosis
Pyroptosis

ABSTRACT

Neutrophils are essential for the innate immune response against bacterial pathogens and play a key role during the early phases of infection, including mastitis and endometritis in cows. When directly challenged with bacteria, neutrophils undergo phagocytosis induced cell death (PICD). The molecular mechanisms of this cell death modality are poorly understood, especially for bovine neutrophils. Therefore, this study aimed to determine the mechanisms and hallmarks of PICD in bovine neutrophils after *in vitro* challenge with *Escherichia coli* (*E. coli*). Our data show that various apoptotic hallmarks such as blebbing, chromatin condensation and executioner caspase (C)-3/-7 activity are only observed during constitutive bovine neutrophil apoptosis. In contrast, bovine neutrophil PICD is characterized by production of reactive oxygen species (ROS), pro-inflammatory C-1 activation, nuclear factor (NF)-κB activation, and interleukin (IL)-1β and IL-6 secretion. Nevertheless, under both conditions these phagocytes undergo cell death with the exposure of phosphatidylserine (PS). Although PS exposure is generally attributed to the anti-inflammatory features of executioner caspase-dependent apoptosis, it surprisingly preceded plasma membrane rupture during bovine neutrophil PICD. Moreover, C-1 inhibition strongly affected IL-1β production but not the PICD kinetics. This indicates that the secretion of the latter pro-inflammatory cytokine is a bystander effect rather than a regulator of PICD in bovine neutrophils, in marked contrast to the IL-1β-dependent pyroptosis reported for macrophages.

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http://dx.doi.org/10.1016/j.vetimm.2013.02.003

1. Introduction

Neutrophils play an essential role in the first line defense against bacterial pathogens. In the absence of inflammation, they rapidly and continuously undergo spontaneous apoptosis. During inflammation, their short lifespan is modified and the expression of various pro-inflammatory cytokines as well as the formation of neutrophil extracellular traps (NETs) are known to prolong either neutrophil survival or function (Luo and Loison, 2008). In combination with neutrophil chemotaxis, this prolongation contributes to increased neutrophil numbers at the site of infection as has been observed for bovine neutrophils in the context of subclinical mastitis (Boutet et al., 2004; Sladek and Rysanek, 2001).

Upon bacterial challenge, neutrophils migrate toward the infectious agent and the phagocytosis of these bacteria preludes cell death programs. In humans and mice, several studies have reported an acceleration of neutrophil apoptosis following pathogen ingestion (Engelich et al., 2001; Perskvist et al., 2002; Rotstein et al., 2000; Sim et al., 2005; Watson et al., 1996). Data on the molecular events during phagocytosis induced cell death (PICD) show that the reactive oxygen species (ROS) generated after a phagocytic stimulus promote the onset of human and mouse neutrophil cell death by triggering phosphatidylserine (PS) exposure (Perskvist et al., 2002; Semiramoth et al., 2010; Sim et al., 2005; Wilkie et al., 2007; Zhang et al., 2003). This outer membrane localization of PS contributes to the recognition and engulfment of the neutrophils by macrophages (Fadeel et al., 1998; Wilkie et al., 2007). However, the contribution of the key executioners of the apoptotic program, caspase-3 (C-3) and C-7, to PICD of neutrophils remains controversial (Fadeel et al., 1998; Kroemer et al., 2009; Perskvist et al., 2002; Wilkie et al., 2007; Zhang et al., 2003).

Reports on PICD in isolated bovine neutrophils are scarce. Moreover, they are typically based on PS exposure and membrane permeability evaluated with propidium iodide (PI) and do not shed light on the underlying molecular mechanisms (Boutet et al., 2004; Chang et al., 2004; Notebaert et al., 2005; Piepers et al., 2009; Sladek and Rysanek, 2011). Nevertheless, it has been observed that stimulation of bovine neutrophils with mastitis pathogens triggers the generation of deconsensed chromatin NETs loaded with antimicrobial components (Grinberg et al., 2008; Lippolis et al., 2006). Interestingly, NET-forming human neutrophils can undergo a caspase-independent neutrophil cell death that is distinct from apoptosis and necroptosis, called NETosis (Fuchs et al., 2007; Remijsen et al., 2011). Human neutrophils undergoing NETosis do not expose the 'eat-me' signal PS prior to plasma membrane rupture (Remijsen et al., 2011), thus preventing their clearance and allowing prioritization of NET generation. Therefore, the pro-inflammatory NETosis cell death modality is not characterized by the anti-inflammatory effects of apoptosis including the display of PS (Brinkmann et al., 2004; Fuchs et al., 2007; Luo and Loison, 2008).

Overall, neutrophil cell death following pathogen elimination is required for the resolution of inflammation, which in turn prevents further host tissue damage (Krysko et al., 2011; Notebaert et al., 2005; Paape et al., 2003). As mentioned, the exposure of PS before plasma membrane rupture is observed during some neutrophil cell death modalities, including apoptosis, but not during all (Luo and Loison, 2008; Notebaert et al., 2005; Remijsen et al., 2011). It serves an important anti-inflammatory function, inducing both the clearance of neutrophils by pro-inflammatory macrophages and subsequently modifying these latter phagocytes into an anti-inflammatory status (Savill et al., 2002).

As the molecular events associated with PICD pathways remain largely unraveled in bovine neutrophils, the current study aimed to characterize the events induced after phagocytosis of life mammary pathogenic *Escherichia coli* in comparison to spontaneous neutrophil apoptosis. More specifically, the differential role of executioner C-3/-7 activation *versus* that of the pro-inflammatory C-1 and nuclear factor (NF)-κB, the neutrophil ROS production and interleukin (IL)-1β/-6 secretion were investigated.

2. Materials and methods

2.1. Isolation of bovine neutrophils

Healthy heifers of the Holstein-Friesian breed were selected from the Ghent University dairy farm (Biocentrum Agri-Vet, Melle, Belgium). Bovine neutrophils were isolated as previously described (Notebaert et al., 2005). Briefly, peripheral blood was collected from the jugular vein using sterile tubes (IMI, Montegrotto Terme, Italy) pre-filled with an equal volume of Alsever anticoagulant solution (3 mM citric acid monohydrate, 27 mM trisodium citrate dihydrate, 72 mM sodium chloride and 125 mM D-glucose, pH 6.1). The blood sampling was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Ghent University. Plasma and the buffy coat were removed following centrifugation at 300 × *g* for 15 min. Erythrocytes were lysed by incubation for 10 min with an ice-cold isotonic NH_4Cl solution (138 mM NH_4Cl and 21 mM Tris, pH 7.4). The remaining cells were washed twice in phosphate buffered saline (PBS) and pelleted at 200 × *g* for 10 min. Contaminating mononuclear cells were then removed by density gradient centrifugation at 1000 × *g* for 20 min using 1.094 g/ml Percoll (Sigma–Aldrich, Bornem, Belgium). The remaining cells were washed twice with PBS. To check neutrophil purity, the cells were stained with Hemacolor (Merck Chemicals Ltd., Nottingham, United Kingdom) and examined under a microscope. Over 98% of the isolated cells were polymorphonuclear granulocytes with less than 5% eosinophils. Viability of the cells was evaluated by incubation with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) as described in Section 2.3. More than 96% of the neutrophils were Annexin V-$FITC^-$/PI^- and thus viable.

2.2. Phagocytosis of E. coli by bovine neutrophils

Isolated neutrophils were resuspended in sterile RPMI 1640 with 10% fetal calf serum (FCS) (both from Invitrogen, Merelbeke, Belgium) at a density of 5×10^6 cells/ml. Live *E. coli* P4:O32, obtained from a bovine mastitis isolate (Bramley, 1976), were added to the neutrophil cultures at

a multiplicity of infection (moi) of 5:1 following overnight growth in Brain Heart Infusion broth (Oxoid, Drongen, Belgium). The moi employed in other *in vitro* studies ranged from 2 to 50 bacteria, yeast particles or latex beads per neutrophil (Choy et al., 2004; Wilkie et al., 2007). Since only a few live bacteria are required to start infection *in vivo*, a relatively low ratio was chosen to more accurately model the clinical situation. Moreover, viable cells instead of heat-inactivated bacteria were used to further promote clinical relevancy. Incubation was performed at 37 °C with mild rotation. Phagocytosis was stopped at 0, 3, 6, 20 and 24 h by placing the cells on ice, and samples were analyzed immediately. In total 8 independent phagocytosis experiments were performed, and for each experiment 2–4 heifers were used.

2.3. PS exposure and cell membrane integrity of bovine neutrophils

To the isolated neutrophils of 5 heifers either no, or 20 μM of the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) (Bachem, Bubendorf, Switzerland), or 50 μM of the C-1 inhibitor Ac-Tyr-Val-Ala-Asp-cho (Ac-YVAD-CHO) (Bachem, Bubendorf, Switzerland), or 10 μM necrostatin-1 (Merck Chemicals Ltd.) was added. Following incubation without or with bacteria at 0, 3, 6 and 20 h, 1×10^6 neutrophils were centrifuged at $200 \times g$ for 10 min and resuspended in incubation buffer (10 mM Hepes, 140 mM sodium chloride and 5 mM calcium chloride, pH 7.4) with Annexin V-FITC labeling reagent (Roche Diagnostics, Vilvoorde, Belgium) and PI (1 μg/ml; Sigma–Aldrich). After incubation for 10 min, PBS was added and samples were analyzed using a FACSCanto™ flow cytometer (Becton Dickinson Biosciences, Erembodegem, Belgium). Ten thousand events were collected and analyzed using the FACSDiva software (Becton Dickinson Biosciences). PS exposure and plasma membrane integrity were evaluated based on the Annexin V-FITC and PI fluorescence. For all 8 experiments performed, this parameter was measured but only the data of the last 2 experiments, where the effect of different inhibitors were additionally evaluated, are shown.

2.4. Morphological analysis of bovine neutrophils

2.4.1. Light microscopy

At early (0.5 h), intermediate (2–6 h) and late (20 h) time points during incubation with or without *E. coli*, 2×10^5 neutrophils were harvested and cytospins were prepared by centrifugation at $55 \times g$ for 5 min. Slides were air-dried, fixed in methanol and stained with Hemacolor (Merck, Darmstadt, Germany). Cytospins were made of neutrophils of minimally 5 heifers from 3 independent phagocytosis experiments.

2.4.2. Live cell imaging

2.4.2.1. GFP-labeled E. coli. The Green Fluorescent Protein (GFP) bacterial plasmid GFP pFPV25.1 has been previously described (Valdivia and Falkow, 1996). The plasmid was isolated from GFP-*Salmonella typhimurium* using the Qiagen plasmid Midi kit (Qiagen, Hilden, Germany). The GFP P4:O32 strain of *E. coli* was generated by electroporation of the parent P4:O32 strain. Briefly, 3.5 ml of log-phase culture of P4:O32 was collected by centrifugation, washed twice in ice-cold water and then resuspended in 40 μl ice-cold water. GFP pFPV25.1 DNA (2 μg) was added to the bacterial suspension, dispensed into a 0.1 cm electroporation cuvette and pulsed at 1.6 kV (Genetronics, Sorrento, San Diego). Immediately following electroporation 500 μl SOC media was added to the bacteria, the bacterial suspension was transferred to a 1.5 ml microfuge tube and incubated at 37 °C for 30 min with shaking. Positive transformants were selected on Luria Bertani (LB) agar plates supplemented with 100 μg/ml of ampicillin.

2.4.2.2. Live cell imaging. Four hundred thousand neutrophils were seeded in eight-well coverglass based chambers (Nalge Nunc International, Rochester, NY, USA). After addition of GFP-*E. coli* (moi 5:1) to the test wells, and of PI to both test and control wells at a final concentration of 3 μM, cells were imaged using a Leica Application Solution Multi-Dimensional Workstation (AS-MDW) equipped with a DM IRE2 microscope with a PIFOC P-Piezo element-driven HCX PL APO 63x/1.3 NA immersion objective, a 75-W Xenon lamp (with monochromator) set at 2 mW, and a 12-bit Coolsnap HQ camera (Leica Microsystems, Wetzlar, Germany). Cell morphology was observed by differential interference contrast (DIC). Fluorescence excitation wavelengths were 489 nm and 533 nm, and BP515-560/FT580/LP590 and BP470/40/FT500/BP525/50 filter cubes were used to detect fluorescence emission of PI and GFP, respectively. Phototoxicity and photobleaching were minimized by limiting fluorescence excitation exposure time (60 ms) by setting the camera at 2×2 binning. Cells were monitored for 8 h in total, and three image stacks (DIC, PI, and GFP) were captured every 2 min. Three positions per well were imaged. Each image stack consisted of 16 images at different focal planes set at 1 μm intervals to prevent loss of focus of the nonadherent cells. From each image stack, maximum intensity projections (for PI and GFP) and autofocus images (for DIC) were made for each time point using a script developed in house for ImageJ 1.31i public domain imaging software. 3D deconvolution (iterative restoration based on calculated PSFs) was performed on Image sequences of PI and GFP using the Velocity software 5.2.0 (Perkin Elmer, Coventry, UK). Subsequent montages of the Multi-tiff time series and three-channel overlays were made in Image J 1.31i. At least 4 independent live cell imaging experiments were performed.

2.5. Caspase-3/-7 activation in bovine neutrophils

Caspase-3/-7 activity was determined in 2 independent experiments. After different incubation times (0, 6 and 24 h) in the absence or presence of bacteria, neutrophils of 6 heifers were centrifuged at $200 \times g$ for 10 min and lysed on ice in caspase lysis buffer (200 mM sodium chloride, 10 mM Tris–HCl pH 7.4, 5 mM EDTA, 10% glycerol and 1% NP-40) supplemented with protease inhibitors (0.15 μM aprotinin, 2.1 μM leupeptin and 100 nM phenylmethylsulfonyl fluoride; all from Sigma–Aldrich) and oxidized glutathione (1 mM, Sigma–Aldrich). After centrifugation at $8000 \times g$

for 10 min, the supernatant was collected and the protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Life Science, Nazareth, Belgium) based on the method of Bradford. To determine C-3/-7 activity, the Caspase-Glo® Assay (Promega, Leiden, The Netherlands) was used according to the manufacturer's protocol. In a 96-well plate, 10 μg of the protein lysate was diluted with lysis buffer to a final volume of 100 μl and an equal amount of Caspase-Glo reagent containing DEVD-aminoluciferin as a substrate for C-3 and -7 was added. After 1 h incubation at room temperature, CL was measured with a luminometer (Fluoroskan Ascent FL, Thermo Fisher Scientific) and expressed in RLU/s.

2.6. Immunoblot analysis of bovine neutrophil lysates

Laemmli buffer (final concentration of 62.5 mM Tris–HCl pH 6.8, 100 mM dithiotreitol (DTT), 2% sodium dodecyl sulfate (SDS), 0.1% bromophenolblue and 10% glycerol) was added to the protein lysates. Samples were boiled for 10 min for complete denaturation before loading. As a positive control for poly(ADP-ribose) polymerase (PARP) cleavage, a commercial ready-to-use cell extract of human HL-60 leukemia cells, induced to undergo apoptosis by the chemotherapeutic agent etoposide (Enzo Life Sciences International, Plymouth Meeting, USA) was included. Bafilomycin (100 nM) treated Ba/F3 WT cells upon IL-3 deprivation and doxorubicine (12 μM) induced intestinal cells (DMBR, VIB, Zwijnaarde, Belgium) were used as a control for microtubule-associated protein 1 light chain (LC) 3–I cleavage and activation of C-3/-7, respectively. As a control for C-1 activation, bone marrow derived macrophages (BMDM) isolated from C57BL/6 mice were incubated for 3 h with 5 μg/ml LPS (Invivogen, San Diego, CA, USA) and transfected with empty plasmid DNA to induce AIM2-inflammasome-mediated C-1 cleavage. Equal amount of lysates (15–25 μg) and of the positive controls (7.5–15 μg) were separated in SDS–polyacrylamide gel electrophoresis (PAGE) gels. To obtain optimal resolution on PAGE, 15% acrylamide gels were used for C-3, C-7 and XIAP, 18% for LC-3 and 12% for C-1 and PARP detection. Next, proteins were transferred to nitrocellulose membranes by semi-dry blotting in a buffer containing 47.9 mM Tris–HCl (pH 8.0), 38.6 mM glycine, 1.4 mM SDS and 20% methanol. Blocking, incubation with antibody and washing of the membrane were all performed in Tris buffered saline (TBS) supplemented with 0.1% Tween-20 (v/v) and 5% (w/v) non-fat dry milk on a platform shaker. To achieve better binding efficiency of C-1 antibody, Tween-20 was omitted. The primary antibodies used were anti-cleaved C-3 (Cell Signaling Technology, Danvers, MA, USA) and C-7 (Cell Signaling Technology, Danvers, MA, USA), X-IAP (MBL, Woburn, MA, USA), PARP (Enzo Life Sciences International, Plymouth Meeting, PA, USA), LC-3 (Sigma–Aldrich, St. Louis, MO, USA) and polyclonal anti-recombinant murine C-1 (Lamkanfi et al., 2004). The anti-PARP antibody recognized intact PARP (116 kDa) as well as its apoptosis-related fragment (85 kDa) and its necrosis-related fragments (50, 62 and 74 kDa). The LC-3B antibody recognized both LC-3B forms, the cytosolic form called LC3-I (18 kDa) and an autophagosome-associating form LC3-II (16 kDa). An antibody against actin (Becton Dickinson Biosciences, Erembodegem, Belgium) was used on all blots to check if samples were equally loaded. Membranes were incubated with horseradisch peroxidase (HRP)-conjugated secondary antibodies against mouse and rabbit immunoglobulin (Amersham Biosciences, Piscataway, NJ, USA). Immunoreactive proteins were visualized by enhanced CL (PerkinElmer, Waltham, MA, USA) and exposed to a film (Amersham Biosciences, Piscataway, NJ, USA). To determine the quantity level of the band ImageJ software was used. Western blot analysis was performed on samples of minimally 5 heifers from 3 independent experiments.

2.7. ROS production by bovine neutrophils

Reactive oxygen species were measured using the chemiluminescence (CL) technique in an independent experiment with 4 heifers. Luminol (Sigma–Aldrich, St. Louis, USA) was used at 200 μM as chemiluminigenic probe. The CL response of bovine neutrophils stimulated with *E. coli* was compared to the response obtained after stimulation with PMA at a concentration of 20 μg/ml (Sigma–Aldrich, St. Louis, USA). Neutrophils were seeded in a white 96-well plate with clear bottom (Greiner Bio-one GmbH, Frickenhausen, Germany) at 1×10^6 cells/well in 100 μl of RPMI + 10% FCS. Neutrophil-free wells and cells without triggering agents (*i.e.* spontaneous CL) were used as negative controls. To suppress neutrophil ROS production, the NADPH oxidase inhibitor diphenyleneiodonium (DPI) (Sigma–Aldrich, St. Louis, USA) was added to half of the wells at a final concentration of 10 μM. The total plate was pre-incubated at 37 °C for 30 min. The CL reaction was started by adding either 1×10^7 bacteria/well (corresponding to 10 bacteria per neutrophil) or by adding PMA (32 μM) as a positive control. The production of ROS was measured during 60 min at 37 °C in a microplate luminometer (Fluoroscan Ascent Fl, Thermo Labsystems, Helsinki, Finland). The CL response was expressed as relative light units/min (RLU/min). All measurements were performed in triplicate.

2.8. IL-1β and IL-6 secretion by bovine neutrophils

Supernatants obtained after pelleting the cultured neutrophils were used to determine secreted pro-inflammatory cytokine levels.

Secreted IL-1β was determined in the neutrophil supernatants of the 2 inhibitor experiments described in Section 2.3 with a specific bovine IL-1β ELISA (Pierce, Rockford, USA). The method was carried out as described by the manufacturer. 100 μl of undiluted supernatant was added per well.

IL-6 in the neutrophil supernatants of 6 heifers of 2 independent experiments with and without *E. coli* but without inhibitors was measured in a bio-assay as a hybridoma growth factor for mouse 7TD1 cells. Cells were cultured for 72 h in medium with different dilutions of the samples. A colorimetric hexosaminidase reaction reflects the number of cells which is related to the amount of IL-6 in the media. As no recombinant bovine IL-6 was commercially available, human reference standards were included in this bio-assay.

Analysis was based on the half-maximal proliferation of the cells.

2.9. NF-κB activation in bovine neutrophils

Cultured neutrophils of 10 cows from 3 independent phagocytosis experiments were centrifuged at 200 × *g* for 10 min and lysed on ice in a radioimmunoprecipitation assay (RIPA) lysis buffer (150 mM sodium chloride, 20 mM Hepes, 2 mM EDTA, 0.5% sodium deoxycholate and 1% NP-40, pH 7) with a cocktail of protease inhibitors (Calbiochem, La Jolla, USA). After centrifugation at 8000 × *g* for 10 min, the supernatant was collected and the protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Life Science, Nazareth, Belgium). To determine the NF-κB p65 activity, the TransAM™ Transcription Factor Assay Kit (Active Motif, Rixensart, Belgium) was used according to the manufacturer's protocol. Ten μg of each protein lysate was incubated for 1 h in a 96-well plate containing an immobilized NF-κB consensus oligonucleotide. After washing, the NF-κB complex bound to the oligonucleotide was identified using the supplied anti-p65 antibody. Addition of a secondary antibody conjugated to HRP allowed a chemiluminescent readout (Fluoroskan Ascent FL; Thermo Fisher Scientific, Zellik, Belgium) expressed in RLU/s.

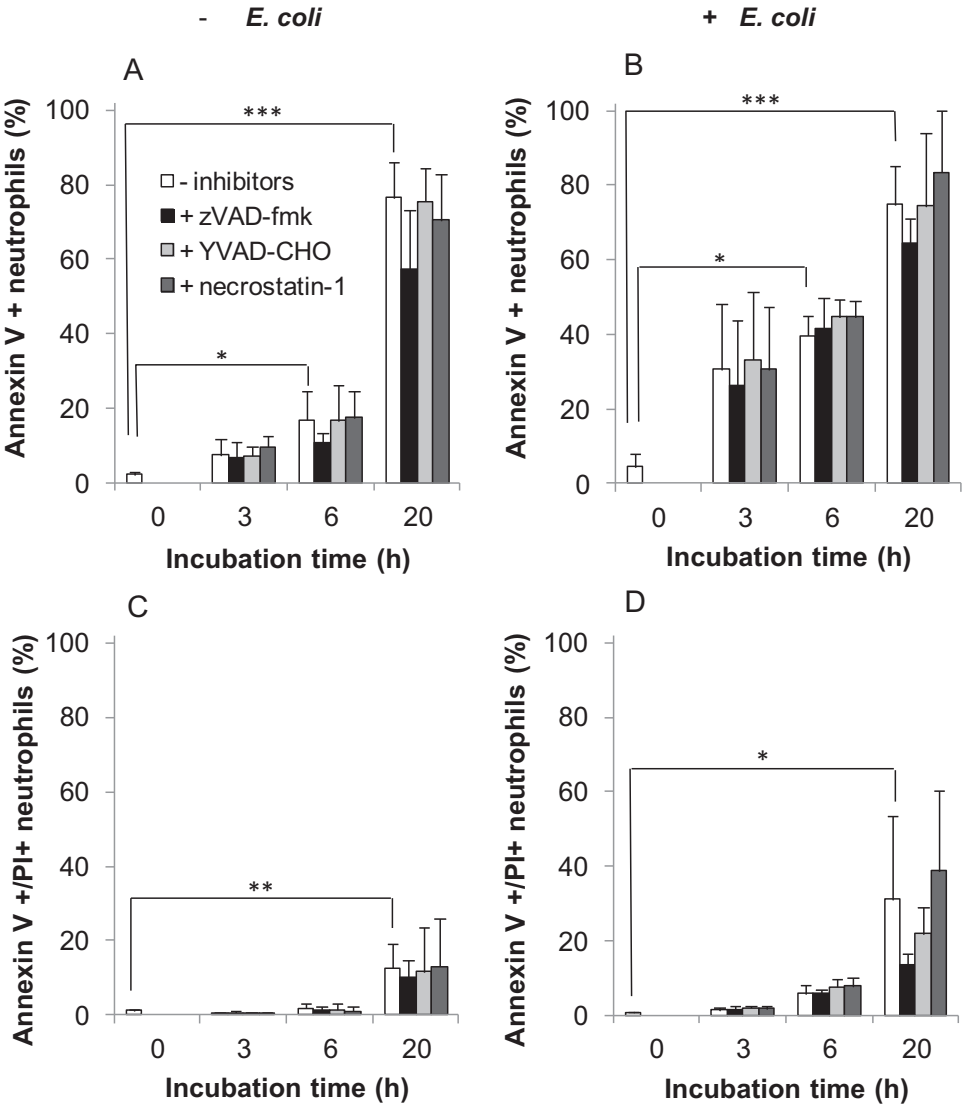

Fig. 1. Percentages of Annexin V$^+$ and of Annexin V$^+$/PI$^+$ isolated bovine neutrophils either treated or untreated with specific inhibitors preceding incubation with or without bacteria. After treatment with or without the inhibitors ZVAD-fmk, YVAD-CHO and necrostatin-1, isolated cells were either stimulated or not stimulated with *E. coli*. These cultured cells were analyzed at 0, 3, 6 and 20 h by flow cytometry after dual staining with Annexin V-FITC and PI as described in Section 2.3. Annexin V$^+$ neutrophils expose phosphatidylserine (PS) on their cell surface after incubation without (A) or with *E. coli* (B). Annexin V$^+$/PI$^+$ neutrophils incubated without (C) or with *E. coli* (D) expose PS and are additionally cell permeable. Data are expressed as means ± SD of 5 heifers obtained from 2 independent inhibitor experiments except for the 0 h time point ($n = 3$). Statistically significant differences are indicated with 1 asterisk ($P < 0.05$), 2 asterisks ($P < 0.01$) or 3 asterisks ($P < 0.001$).

2.10. Statistical analysis

Data which are distributed normally are expressed as mean ± standard deviation (SD) and non-parametric data as median (range). Since ROS data of all groups were distributed normally, a multifactorial model was used to evaluate the effect of ROS production after both *E. coli* and PMA stimulation of bovine neutrophils and after the addition of DPI within each sample group. The IL-1β results and the arcsine transformed Annexin V-FITC and Annexin V-FITC/PI percentages of the different inhibitor conditions were statistically analyzed in a mixed model. The IL-6 and NF-κB data were non-homoscedastic and differences between neutrophils cultured in the absence or presence of *E. coli* bacteria were therefore statistically examined by the non-parametric Wilcoxon matched pairs test. The SPSS Software (SPSS Belux, Brussels, Belgium) was used for all statistical analyses.

3. Results

3.1. E. coli stimulation enhances cell death of bovine neutrophils

Isolated cells were incubated with or without *E. coli* and preceded either with or without treatment of a cell death inhibitor. Exposure of the 'eat-me' signal PS was analyzed by the fluorescence of membrane bound Annexin V-FITC (Fig. 1A and B) and plasma membrane integrity loss analyzed by fluorescence of PI (Fig. 1C and D) using dual staining flow cytometry at 0, 3, 6 and 20 h. Cells that were Annexin V^+ all expose PS, while plasma membrane rupture was detected as Annexin V^+ cells became additionally PI^+.

In the absence of *E. coli*, 17.0 ± 7.6% of cells were Annexin V^+ at 6 h (Fig. 1A). After 20 h, 76.6 ± 9.6% of the cells were Annexin V^+ (Fig. 1A), while only 12.6 ± 6.7% of the total cell population was also PI^+ at that time (Fig. 1C). The pan-caspase inhibitor zVAD-fmk had a visual albeit non-significant suppressive effect on this spontaneous neutrophil apoptosis at both 6 h and 20 h, whereas the two other cell death inhibitors YVAD-CHO and necrostatin-1 had no effect on these cell death readouts (Fig. 1A).

Stimulation with *E. coli* resulted in a markedly ($P < 0.001$) enhanced rate of PS exposure at 3 and 6 h compared to unstimulated neutrophils, with 39.6 ± 5.5% Annexin V^+ cells at 6 h (Fig. 1A *versus* B). However, cells remained largely PI^- at those early time points, and after 20 h only 31.1 ± 22.5% of the total cell population was PI^+ (Fig. 1D). These data indicate that cells expose PS, a hallmark of apoptosis, prior to plasma membrane rupture. This pattern was seen in all independent phagocytosis experiments performed (data not shown). The pan-caspase inhibitor zVAD-fmk and the two other cell death inhibitors did not significantly delay *E. coli* PICD in isolated bovine neutrophils (Fig. 1B and D) suggesting that this induced cell death occurs in a caspase-independent manner.

3.2. E. coli PICD in bovine neutrophils reveals chromatin de-condensation and a dynamic plasma membrane

Shortly after isolation (data not shown) and at 0.5 h of culture with and without bacteria, control and phagocytic neutrophils could be readily detected with their typical polymorphonuclear nucleus (Fig. 2A and D). After 6 h of culture in the absence of *E. coli*, most bovine neutrophils still maintained their multi-lobulated nuclear shape. At that early time point, only few of them had lost this characteristic shape and the latter minority of cells instead displayed a spherical, condensed chromatin structure typical for cells undergoing caspase-dependent apoptosis (Fig. 2B). At the late time point of 20 h, the apoptotic nuclear morphology was observed in the majority of unstimulated neutrophils (Fig. 2C).

In marked contrast, already from 2 h after incubation with *E. coli* these stimulated neutrophils lost their characteristic multi-lobulated nuclei but now no spherical nuclear condensation could be observed. Instead, cells displayed a de-condensed nucleus (Fig. 2E). These features became apparent in all bovine neutrophils cultured with bacteria for up to 20 h (Fig. 2F).

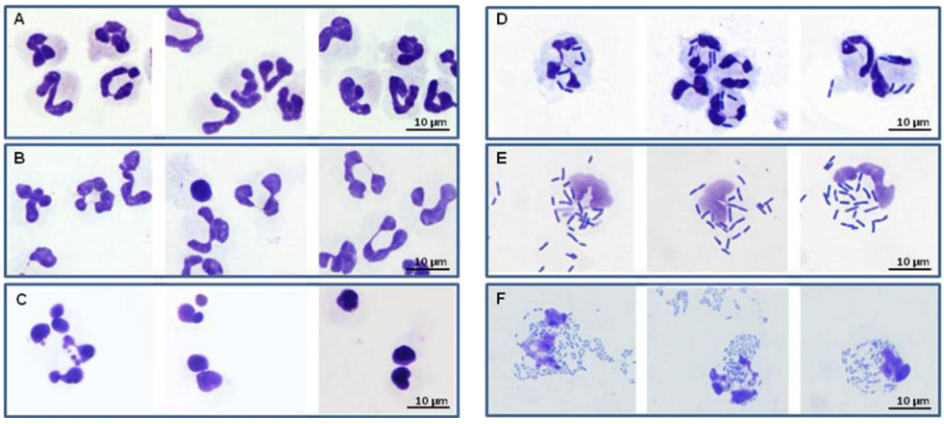

Fig. 2. Representative cytocentrifuge preparations of isolated bovine neutrophils following incubation with or without bacteria. Following cytocentrifugation and Hemacolor staining as described in Section 2.4.1. Chronological images were made at a magnification of 1000× from cultured cells either without (A–C) or with *E. coli* (D–F) at early (0.5 h), intermediate (2–6 h) and late stages (20 h), respectively. Results are representative for minimally 5 heifers from 3 independent experiments.

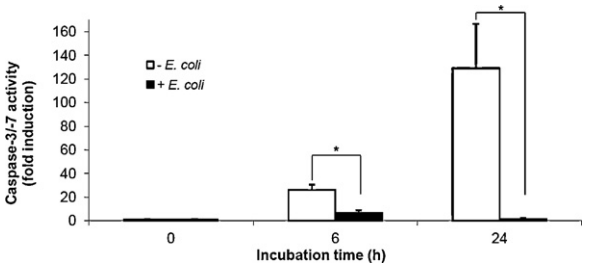

Fig. 3. Caspase-3/-7 activity in isolated bovine neutrophils following incubation with or without bacteria. At 0, 6 and 24 h of incubation with or without *E. coli*, neutrophil lysates were prepared. Caspase-3/-7 activity of the supernatant was measured as described in Section 2.5. The ratio of the measured values for stimulated samples over those of the corresponding control samples at 0 h (fold induction) was determined. Data are expressed as means ± SD of 6 heifers used in 2 independent phagocytosis experiments. Statistically significant differences are indicated with 1 asterisk ($P < 0.05$).

Complementary to light microscopy and at first for neutrophils, live cell imaging was performed to investigate their fate undergoing *E. coli* PICD. For this purpose, neutrophils were incubated with GFP-labeled life mammary pathogenic *E. coli* to visualize phagocytosis, and in addition to morphological changes, cell permeability/death was monitored by the cell impermeable DNA binding fluorochrome PI. In both unstimulated (Supplementary movie 1) and *E. coli* stimulated conditions (Supplementary movie 2), many neutrophils displayed very dynamic plasma membrane morphology. This allowed the distinction between apoptotic blebbing without formation of apoptotic bodies as seen in unstimulated cells (Supplementary movie 1), and chemokinesis and phagocytosis without membrane blebbing in *E. coli* stimulated cells (Supplemental movie 2). Unstimulated neutrophils appeared normal during approximately the first 5 h of imaging, after which the number of PI^+ cells gradually increased. Supplementary movie 2 shows that the uptake of GFP-labeled *E. coli* by bovine neutrophils occurred without any loss of membrane integrity for about 4 h after co-incubation. However, many PI^+ cells were observed when the number of multiplying bacteria became too high.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2013.02.003.

3.3. *E. coli* PICD in bovine neutrophils is independent of executioner C-3/-7 activation

No typical apoptotic morphologic features were observed on bovine neutrophils stimulated with *E. coli*, despite early PS exposure before plasma membrane rupture. To evaluate the contribution of executioner C-3/-7 their activity was analyzed by DEVDase assays (Fig. 3). In the absence of *E. coli*, freshly isolated cells showed minimal caspase activity, whereas 6 h later these values reached significantly ($P < 0.05$) higher levels (Fig. 3). The activation of executioner caspases thus clearly preceded the PS exposure observed during spontaneous neutrophil apoptosis (Fig. 1A). Subsequently, the DEVDase activity further increased significantly ($P < 0.05$) in function of time until 24 h in the absence of *E. coli* (Fig. 3). In marked contrast, cells incubated with bacteria maintained the basal levels of freshly isolated neutrophils for up to 24 h of culture.

In line with these DEVDase results, the cleavage fragments of C-3 were only detected by western blotting during spontaneous bovine neutrophil apoptosis at 24 h (Fig. 4A). Likewise, after 6 h of culture, cleaved C-7 fragments were observed in unstimulated but again not in *E. coli* stimulated neutrophils (Fig. 4A). The intensities of both executioner caspase fragments further increased in time, again exclusively during spontaneous apoptosis. Only after 24 h of stimulation with *E. coli*, a weak signal of cleaved C-7 was seen also in stimulated bovine neutrophils (Fig. 4A). However, the low abundance of these C-7 fragments was insufficient to concomitantly increase the DEVDase activity (Fig. 3).

The cleavage of PARP was next analyzed by western blotting to further assess the proteolytic activity of executioner caspases. Uncleaved PARP has a MW of 116 kDa and is known to be cleaved during apoptosis forming fragments of 89 kDa and 27 kDa. In bovine neutrophils, the expected classical 89 kDa fragment could not be detected either in unstimulated or in *E. coli* stimulated cells (Fig. 4A). Instead, a smaller PARP fragment of about 70 kDa was observed in freshly isolated cells. The levels of this alternative PARP fragment increased during spontaneous neutrophil apoptosis and even more so during *E. coli* PICD (Fig. 4A). At 24 h, *E. coli* stimulated cells showed a substantially stronger signal of this alternatively cleaved PARP fragment. It has to be remarked that although the anti-PARP antibody should also recognize uncleaved PARP of 116 kDa, no signal was detected at this MW. Caspase activity is not only determined by the formation of active caspase but also by the levels of the natural cellular caspase inhibitors. The level of X-linked inhibitor of apoptosis (X-IAP), a known endogenous inhibitor of C-3 and -7, was therefore also analyzed by western blotting. A weak albeit visible X-IAP band was detected in freshly isolated cells and during spontaneous apoptosis, but decreased below the detection limit during *E. coli* PICD in bovine neutrophils (Fig. 4A).

3.4. *E. coli* PICD in bovine neutrophils correlates with ROS production

Since ROS formation is generally associated with (neutrophil) cell death following stimulation, it was investigated to which extent ROS production was induced during *E. coli* PICD compared to spontaneous bovine neutrophil apoptosis.

Unstimulated cells displayed low basal ROS levels, whereas exposure of bovine neutrophils to *E. coli* resulted in significantly ($P < 0.01$) higher ROS levels (Fig. 5). It is hypothesized that *E. coli* activates NADPH oxidase to produce the observed ROS. As a control, PMA, a known activator of NADPH oxidase, was used. PMA stimulation caused a significant ($P < 0.01$) increase in ROS levels similar to *E. coli* and this activation could be significantly ($P < 0.01$) inhibited by pretreatment with the NADPH oxidase inhibitor DPI. Importantly, pretreatment with the latter inhibitor also significantly ($P < 0.01$) reduced the *E. coli* induced ROS formation to basal levels (Fig. 5).

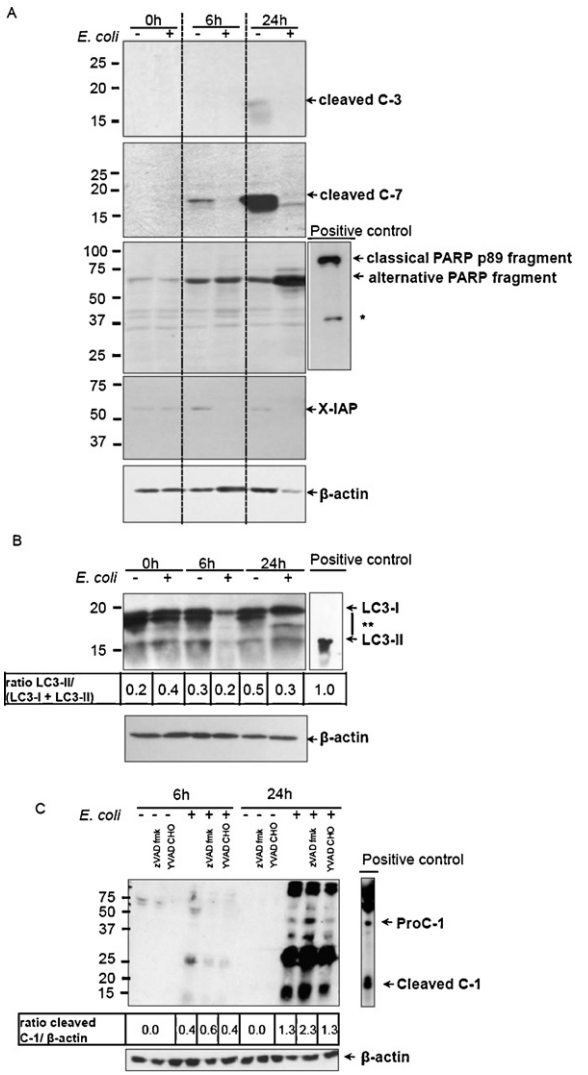

Fig. 4. Western blot analysis of isolated bovine neutrophil lysates following incubation with or without bacteria. At 0, 6 and 24 h of incubation without or with *E. coli*, neutrophil lysates were prepared. Western blot analysis was carried out as described in Section 2.6. Blots were incubated with (A) anti cleaved C-3, anti cleaved C-7, anti PARP and anti X-IAP, (B) anti LC3B and (C) anti recombinant C-1. As a positive control for PARP cleavage a commercial cell extract of etoposide induced human HL-60 leukemia cells was included. An extract of BMDM isolated from C57BL/6 mice incubated with 5 μg/ml LPS and transfected with empty plasmid DNA was used for a positive control of C-1 activation. Lysates of bovine neutrophils pretreated with 20 μM of pan-caspase inhibitor zVAD-fmk or 50 μM of C-1 inhibitor YVAD-CHO were included to evaluate C-1 inhibition. Results are representative for minimally 5 heifers from 3 independent experiments. Anti β-actin was used in all experiments to check cell lysates were equally loaded. The density of cleaved C-1 normalized to the loading control β-actin and the LC3-II/(LC3-II + LC3-I) ratio as an indicator of autophagy were calculated to compare samples. Arrows indicate the fragments of interest. *An aspecific band recognized by the anti PARP antibody. **Presumed intermediate forms of LC3.

3.5. *E. coli* PICD in bovine neutrophils does not convert LC3

As mentioned in Section 1, a caspase-independent cell death modality that requires ROS production is

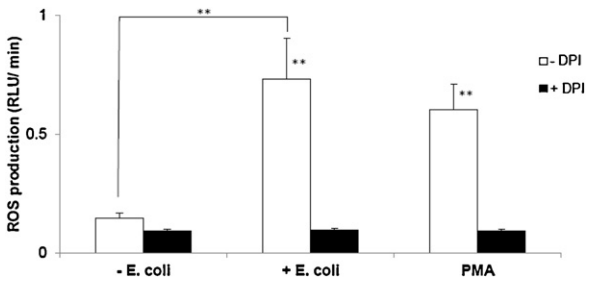

Fig. 5. ROS production in isolated bovine neutrophils following incubation with or without bacteria. Phorbol 12-myristate 13-acetate (PMA) stimulated and diphenyleneiodonium chloride (DPI, a NAPDH oxidase inhibitor) treated neutrophils served as positive and negative controls for ROS production, respectively. Chemiluminescent response after luminol addition was measured for 60 min as described in Section 2.7. ROS production is shown as RLU/min. Data are expressed as means ± SD of 4 heifers obtained from 1 experiment. Statistically significant differences are indicated with 2 asterisks ($P < 0.01$).

NETosis. Although our morphological data did not show NET formation (Fig. 2 and both the supplementary movies), it was still investigated in a confirmatory experiment whether *E. coli* induced NETosis of bovine neutrophils. It was recently shown that NETosis requires the combined induction of both ROS production and autophagy (Remijsen et al., 2011). Therefore, the levels of the autophagy marker LC3 were analyzed. More specifically, the conversion of microtubule-associated LC3-I (p18) to an autophagosome-associated form LC3-II (p16) was assessed by western blotting (Fig. 4B). The positive control for autophagy showed a strong p16 signal and no p18 band. Upon morphometry, a LC3-II/(LC3-II + LC3-I) ratio of 1.0 was thus obtained (Fig. 4B). In the bovine neutrophil lysates, only a weak p16 signal was detected at each time point, regardless of whether cells were activated with *E. coli* or not. This yielded far lower ratio values (<0.5) than the 1.0 value for the positive control. Also, no ratio increase was seen in comparison to control samples at 0 h (Fig. 4B), indicating that although basal housekeeping autophagy occurs, *E. coli* does not induce any additional autophagy in bovine neutrophils.

3.6. *E. coli* PICD in bovine neutrophils induces a pro-inflammatory response

3.6.1. *E. coli* stimulation induces cleavage of C-1 and secretion of IL-1β

Pyroptosis is an executioner caspase- and autophagy-independent cell death modality that differs both biochemically and morphologically from other forms of cell death. More specifically, it strongly depends on C-1 activation and IL-1β secretion. It was therefore analyzed with western blotting whether C-1 activation occurs during *E. coli* PICD in bovine neutrophils. No cleaved C-1 fragments were detectable either in freshly isolated neutrophils or in neutrophils undergoing spontaneous apoptosis until 24 h (Fig. 4C). Morphometry confirmed this observation, yielding a cleaved C-1/β-actin loading control ratio of 0.0. In marked contrast, an amount of cleaved C-1 was generated during the first 6 h after stimulation with *E. coli* further increasing until 24 h (Fig. 4C). The

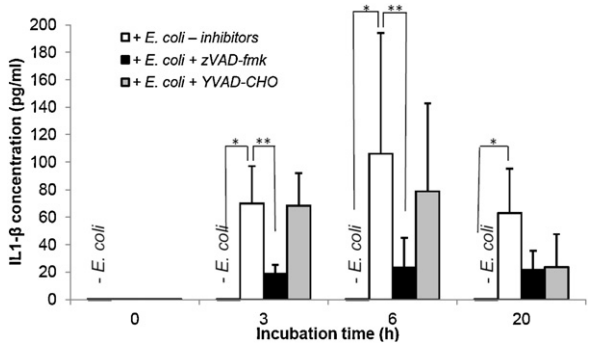

Fig. 6. Concentrations of IL-1β secreted by isolated bovine neutrophils untreated or treated or non-treated with caspase-inhibitors preceding incubation with or without bacteria. Neutrophils were treated or untreated with the pan-caspase inhibitor zVAD-fmk or the C-1 inhibitor YVAD-CHO followed by incubation without or with *E. coli*. Supernatants after 0, 3, 6 and 20 h incubation were collected and IL-1β was quantified using a commercial bovine ELISA. Data are expressed as means ± SD of 5 heifers used in the 2 inhibitor experiments. From these same 5 heifers the data of Annexin V^+ and of Annexin V^+/PI^+ data in Fig. 1 were obtained. Statistically significant differences are indicated with 1 asterisk ($P < 0.05$) or 2 asterisks ($P < 0.01$).

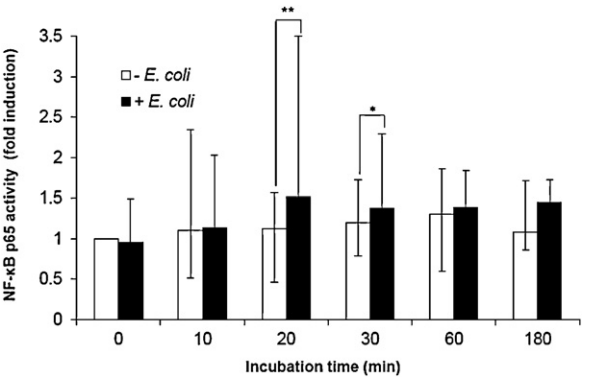

Fig. 7. NF-κB p65 activity in isolated bovine neutrophils following incubation with or without bacteria. At 0, 10, 20, 30, 60 and 180 min of incubation with or without *E. coli*, neutrophil lysates were prepared. NF-κB p65 activity was measured as described in Section 2.9. The activity for the samples were normalized to those of the corresponding control samples at 0 h (fold induction). Data are expressed as medians (range) of 10 heifers obtained from 3 independent experiments. Statistically significant differences are indicated with 1 asterisk ($P < 0.05$) or 2 asterisks ($P < 0.01$).

corresponding morphometry data showed an increasing ratio which was still below 1.0 at 6 h but superseded 1.0 at 24 h. As C-1 activation is typically associated with IL-1β secretion, IL-1β levels were analyzed by ELISA in the supernatants of bovine neutrophils (Fig. 6). No secreted IL-1β was detected at any time point in the absence of *E. coli*. Again in marked contrast, IL-1β levels were significantly increased compared to unstimulated neutrophils as soon as 3 h following initiation of *E. coli* PICD ($P < 0.05$).

Cells were incubated with either the pan-caspase inhibitor zVAD-fmk or the more selective C-1 inhibitor YVAD-CHO to evaluate whether this inhibition decreases C-1 mediated IL-1β secretion. No effect of both caspase inhibitors on the formation of cleaved C-1 in *E. coli* stimulated neutrophils was observed (Fig. 4C). However, treatment of bovine neutrophils with zVAD-fmk followed by stimulation with *E. coli* resulted in a significant inhibition of C-1 mediated IL-1β secretion at 3 h ($P < 0.01$) and at 6 h ($P < 0.01$), whereas YVAD-CHO only resulted in a visual albeit non-significant suppressive effect at the late time points of 6 h and 20 h.

3.6.2. *E. coli* stimulation induces activation of NF-κB and secretion of IL-6

Since phagocytosis-induced ROS can rapidly activate the transcription factor NF-κB, it was analyzed whether NF-κB is activated during *E. coli* PICD in bovine neutrophils. The activity of NF-κB p65 was analyzed with ELISA as previously described by our group (Notebaert et al., 2005) at different early time points (0, 10, 20, 30, 60 and 180 min) following incubation with or without *E. coli* (Fig. 7). No significant changes in NF-κB activity were seen for unstimulated neutrophils, whereas cells incubated with *E. coli* for 20 and 30 min displayed a significant ($P < 0.01$ and $P < 0.05$, respectively) though transient increase in NF-κB p65 activity.

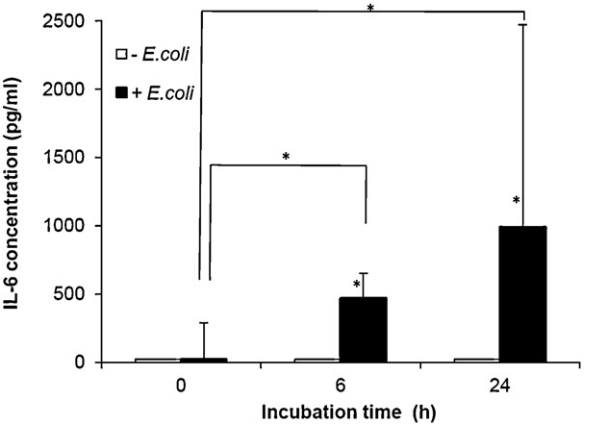

Fig. 8. Concentrations of secreted IL-6 by bovine neutrophils following incubation with or without bacteria. Supernatants of neutrophils at 0, 6 and 24 h of incubation with or without *E. coli* were analyzed with a bio-assay as described in Section 2.8. Data are expressed as medians (range) of 6 heifers used in 2 independent phagocytosis experiments. Concentrations of IL-6 in all samples without *E. coli* were below the detection limit. Statistically significant differences are indicated with 1 asterisk ($P < 0.05$).

As the pro-inflammatory cytokine IL-6 is a read-out of NF-κB activation, the secretion of IL-6 was evaluated at 0, 6 and 24 h in the supernatants of bovine neutrophils incubated with or without *E. coli* by a bio-assay (Fig. 8). No IL-6 was detected at any time point in the supernatants of unstimulated cells. In contrast, *E. coli* clearly induced ($P < 0.05$) IL-6 secretion. As soon as 6 h after *E. coli* stimulation IL-6 levels were abundant, with a further significant increase ($P < 0.05$) until 24 h.

4. Discussion

Coliform pathogens are involved in several important infectious diseases in cattle, such as endometritis and mastitis (Bradley, 2002; Dadarwal et al., 2007). As a main characteristic of the dairy cow's innate immune response,

neutrophils phagocytose the invading pathogens leading to a complex cascade of molecular events at the site of infection. Generally, but especially in the bovine, these events have scarcely been investigated, in contrast to the well-studied preceding processes of neutrophil chemotaxis and migration (Sladek and Rysanek, 2011). This study therefore aimed to characterize the cell death mechanism of bovine neutrophils following *in vitro* phagocytosis of live mammary pathogenic *E. coli* at the molecular as well as morphological level.

Although an enhanced rate of PS exposure was observed, *E. coli* PICD markedly differed from spontaneous neutrophil apoptosis (Table 1) because it occurred in the absence of the executioner C-3 cleavage, was caspase inhibition insensitive, totally lacked DEVDase activity, and did not show any chromatin condensation or membrane blebbing. Caspase-7 cleavage was also profoundly reduced until 6 h, still some C-7 fragmentation was detected at later time points. Given the sensitivity of the DEVDase readout, the lack of any proteolytic activity underscored the executioner C-3/-7 independency of *E. coli* PICD in isolated bovine neutrophils. Our results further showed that X-IAP, the well-described natural inhibitor of both active C-3 and C-7, was only present in unstimulated neutrophils. This indicated that X-IAP may control the C-3/-7 activity observed in spontaneous bovine neutrophil apoptosis but is superfluous in *E. coli* PICD, at least *in vitro*. As Wilkie et al. (2007) have demonstrated that functional NAPDH oxidase and the generation of ROS prevent the activation of the cytoplasmic caspase cascade in both human and mouse neutrophils following phagocytosis of *Staphylococcus aureus*, we hypothesize that not X-IAP but the induced NADPH oxidase derived ROS production is responsible for the lack of C-3/-7 activity in *E. coli* PICD of bovine neutrophils. Furthermore, ROS are known to promote PS exposure (Kagan et al., 2003) and anti-oxidants can inhibit the latter flip-flop process (Tyurina et al., 2004). It is therefore conceivable that the LPS-TLR4 induced ROS production by bovine neutrophils in the current study also mediated the PS exposure during *E. coli* PICD.

The *E. coli* PICD also clearly differed from NETosis (Table 1), because besides the common feature of ROS production no LC-3 cleavage was observed, nor was massive and sustained autophagy-dependent vacuolization. Moreover, PS exposure and plasma membrane rupture occur simultaneously during NETosis of human neutrophils (Remijsen et al., 2011), whereas PS exposure preceded *E. coli* PICD in isolated bovine neutrophils. Our data therefore indicate that *E. coli* PICD in bovine neutrophils is autophagy-independent and thus differs from NETosis. Nevertheless, Lippolis et al. (2006) and Grinberg et al. (2008) described NETs formation by bovine blood neutrophils following exposure to *E. coli*. The discrepancy between these results and our data can at least partially be explained by *E. coli* strain differences and the age, lactation stage and parity of the cows. Additionally, both latter studies evaluated the anti-bacterial aspects rather than the PICD mechanism as characterized in the current study. Moreover, for this purpose they only assessed morphological changes induced by *E. coli* in isolated bovine neutrophils, while we focused on the molecular changes. Therefore, Lippolis et al. (2006) and Grinberg et al. (2008) observed NETs formation but did not actually prove that NETosis occurred as *E. coli* PICD modality in bovine neutrophils.

The *E. coli* PICD also differed from receptor-interacting serine/threonine-protein kinase 1 (RIPK1)-dependent programmed necrosis (necroptosis, Table 1), again because PS exposure preceded plasma membrane rupture. Additionally, the RIPK1 inhibitor necrostatin-1 did not influence either the bovine neutrophil cell death hallmarks or kinetics. Common events with the necroptosis cell death modality were again ROS production but also the secretion of the pro-inflammatory cytokine IL-6. The latter could be causally linked to the well-known LPS-TLR4 complex activation of NF-κB signaling which was at first observed by our group upon bovine neutrophil *E. coli* stimulation (Notebaert et al., 2005). However, NF-κB activation has been described to require the adaptor function of RIPK1 and not its kinase activity (Bertrand et al., 1993).

Finally, the *E. coli* PICD also differed from pyroptosis (Table 1), a cell death modality typically observed in macrophages and at first investigated in neutrophils in the current study. This could be concluded mainly because inhibition of C-1 reflected in the subsequent inhibition of the matured IL-1β secretion, did not affect the kinetics of *E. coli* induced PICD. It should be remarked that the irreversible pan-caspase inhibitor zVAD-fmk was more successful at inhibition of C-1 activity compared to

Table 1
Overview of the observed features of *E. coli* PICD in isolated bovine neutrophils compared to reported cell death types in human PICD (apoptosis, NETosis, necroptosis and pyroptosis). If a characteristic of a cell death type in human PICD was observed in *E. coli* PICD of bovine neutrophils this is symbolized with "+". If a characteristic of a cell death type was not observed in *E. coli* PICD of bovine neutrophils (*i.e.* preceded by "No"), this is symbolized with "–".

Features of *E. coli* PICD in bovine neutrophils	Cell death types			
	Apoptosis	NETosis	Necroptosis	Pyroptosis
PS exposure prior to membrane rupture	+		–	
No spherical nuclear condensation or blebbing	–			
No massive vacuolization		–		
No cleaved C-3/-7 nor C-3/-7 activity	–			
Early ROS production		+	+	
No increase in LC-3 cleavage (autophagy)		–		
Cleaved C-1 prior to IL-1β secretion				+
No effect of caspase inhibition on PS exposure				–
NF-κB activity prior to IL-6 secretion			+	
No effect of necrostatin-1 on PS exposure			–	

the presumed more specific but reversible C-1 inhibitor YVAD-CHO. This intriguing observation corroborates earlier data from several authors on the varying degree of effectiveness of these caspase-inhibitors (Luo and Loison, 2008). Regardless, neither of both inhibitors delayed *E. coli* PICD. The latter cell death modality being executioner C-3/-7-independent was thus overall insensitive to caspase-inhibition and to C-1 inhibition in particular. These data indicate that activation of C-1 is a bystander event during *E. coli* PICD, rather than an essential mediator of the latter cell death in bovine neutrophils, in marked contrast to the IL-1β-dependent pyroptosis reported for macrophages. We hypothesize that the activation of proC-1 is probably inflammasome-induced in bovine neutrophils, in analogy with the recently described LPS-stimulated mouse neutrophil data (Lu et al., 2012). Importantly, both these related pro-inflammatory signaling events are apparently not essential for the execution of the neutrophil cell death. We significantly extended our earlier data that *E. coli* PICD in bovine neutrophils is associated with very a NF-κB dependent transcription of pro-inflammatory cytokines including proIL-1β, thus corroborating literature in human and mouse neutrophils (Notebaert et al., 2005). We found that this initial LPS-TLR4 mediated signaling is followed by C-1 mediated maturation of proIL-1β to IL-1β within a few hours.

A final surprising finding of the current study was the accumulation of an atypical PARP fragment of 70 kDa during both spontaneous and *E. coli* PICD. It is tempting to suggest the formation of an atypical cleavage by non-oxidative burst dependent on calpains or granzymes, as suggested by Malireddi et al. (2010). However, the expected uncleaved PARP was not detected by the anti-PARP antibody in the neutrophil lysates, and was moreover absent in the positive control too. We can therefore not rule out the possibility that bovine neutrophils induce the expression of an alternatively spliced PARP fragment during cell death, rather than inducing an alternative cleavage.

In summary, our data indicate that *E. coli* PICD is independent of apoptotic executioner caspase-3/-7 and of the inflammatory caspase-1 but with NF-κB activation, ROS and pro-inflammatory cytokine production, and PS exposure prior to membrane rupture. Surprisingly, stimulation with *E. coli* did not induce autophagy as previously reported for human neutrophils (Mitroulis et al., 2010). However, no clearly defined other cell death modality was observed. Indeed, when the observed characteristics were compared to those of reported cell death types in human and mouse PICD (Galluzzi et al., 2012; Melino et al., 2005), *E. coli* PICD in isolated bovine neutrophils seemed to share features with several of these cell death mechanisms, but nevertheless systematically differed with other features from each of those previously described modalities (summarized in Table 1).

By using this yet undefined cell death modality, the dairy cow's main early defense leukocyte combine on the one hand a pro-inflammatory innate immune response by induction of ROS-dependent antimicrobial strategies and secretion of IL-1β/-6 following *E. coli* stimulation, while on the other hand ensuing an anti-inflammatory response by accelerating PS exposure ("eat me"-signal) before plasma membrane rupture. This *in vitro* bovine neutrophil cell death modality has not yet been described and should be further explored to gain better understanding in its relevance for the host. Yet, it is hypothesized to facilitate the resolution of the inflammatory process following the potent anti-microbial action of neutrophils through their elimination by surrounding macrophages, thus minimizing tissue damage (Savill et al., 2002).

Conflict of interest statement

All authors declare they have no financial or commercial conflicts of interest.

Acknowledgments

The authors wish to thank Wilma Burm for IL-6 analyses, Stanislas Sys and Jella Wauters for statistical support. Eef Parthoens performed excellent technical assistance in live cell imaging and Donna Vanhauteghem assisted during the inhibitor experiments. The authors wish also to thank Ellen Wirawan and Kristof Kersse for their interpretation on the results and helpful hints. Isabel Kalmar helped to add the reference list *via* Endnote. The *E. coli* strain was a kind gift from Christian Burvenich. Quinten Remijsen holds a postdoctoral mandate position from the 'Fonds voor Wetenschappelijk Onderzoek'. This work was financially supported by the Research Foundation-Flanders (research program B/06931/0101). Research in the Vandenabeele group is supported by European grants (FP6 ApopTrain, MRTN-CT-035624; FP7 EC RTD Integrated Project, Apo-Sys, FP7-200767; Euregional PACT II), Belgian grants (Interuniversity Attraction Poles, IAP 6/18), Flemish grants (Research Foundation Flanders, FWO G.0875.11 and FWO G.0973.11), Ghent University grants (MRP, GROUP-ID consortium) and grants from Flanders Institute for Biotechnology (VIB). P.V. holds a Methusalem grant (BOF09/01M00709) from the Flemish Government. The study sponsors had no involvement at any level of the manuscript development including the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication. We thank Dr. Amin Bredan (DMBR-VIB, Ghent) for editing the manuscript.

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