Bovine neutrophils release extracellular traps and cooperate with macrophages in *Mycobacterium avium* subsp. *paratuberculosis* clearance

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Abstract

Mycobacterium avium subsp. *paratuberculosis* (Map) is the underlying pathogen causing bovine paratuberculosis (PTB), an enteric granulomatous disease that mainly affects ruminants and for which an effective treatment is needed. Macrophages are the primary target cells for Map, which survives and replicates intracellularly by inhibiting phagosome maturation. Neutrophils are present at disease sites during the early stages of the infection, but seem to be absent in the late stage, in contrast to healthy tissue. Although neutrophil activity has been reported to be impaired following Map infection, their role in PTB pathogenesis has not been fully defined. Neutrophils are capable of releasing extracellular traps consisting of extruded DNA and proteins that immobilize and kill microorganisms, but this mechanism has not been evaluated against Map. Our main objective was to study the interaction of neutrophils with macrophages during an *in vitro* mycobacterial infection. For this purpose, neutrophils and macrophages from the same animal were cultured alone or together in the presence of Map or *Mycobacterium bovis* Bacillus-Calmette-Guérin (BCG). Extracellular trap release, mycobacteria killing as well as IL-1β and IL-8 release were assessed. Extracellular trap

formation was highest in neutrophils against Map in the presence of macrophages, but without direct cell contact, indicating a paracrine activation. Macrophages were extremely efficient at killing BCG, but ineffective at killing Map. In contrast, neutrophils showed similar killing rates for both mycobacteria. Co-cultures infected with Map showed the expected killing effect of combining both cell types, whereas co-cultures infected with BCG showed a potentiated killing effect beyond the expected one, indicating a potential synergistic cooperation. In both cases, IL-1β and IL-8 levels were lower in co-cultures, suggestive of a reduced inflammatory reaction. These data indicate that cooperation of both cell types can be beneficial in terms of decreasing the inflammatory reaction while the effective elimination of Map can be compromised. These results suggest that neutrophils are effective at Map killing and can exert protective mechanisms against Map that seem to fail during PTB disease after the arrival of macrophages at the infection site.

Introduction

Mycobacterium avium subsp. *paratuberculosis* (Map) is the aetiological agent of paratuberculosis (PTB; Johne's disease), a chronic granulomatous enteritis of ruminants characterized by cachexia and severe diarrhoea resulting in production losses due to silent sub-clinically infection and animal losses due to clinically active disease (1). Infected animals in the subclinical stage shed Map intermittently to the environment, making its control difficult. Vaccination against PTB has been shown to be a cost effective method (2) that reduces shedding and therefore in-herd transmission (3). However, vaccination does not provide complete protection from infection (4). Furthermore, due to the nature of Map antigens, available inactivated vaccines interfere with the official tests for bovine tuberculosis (bTB) eradication program. In this sense, further research on PTB pathogenesis and vaccine development is needed.

Classically, PTB research has been focused on the action of macrophages (MΦ) since these are considered to be the main target cell in the host. After infection, Map is able to survive and replicate intracellularly by inhibiting phagosome maturation. Less attention has been paid to the role that polymorphonuclear neutrophils (PMN) may play in this process, although their presence has been shown at disease sites during the early stages of the infection (5). PMNs are short-lived effector cells capable of phagocytosing

66 pathogens, capturing and killing microbes through the production of reactive oxygen
67 species (ROS), lytic enzymes with potent antimicrobial activity (6) as well as the
68 release of extracellular traps (ETs) in the extracellular space (7, 8).
69
70 ET formation has been demonstrated as an important novel effector mechanism against
71 pathogens in PMNs (8) and MΦs (9), among other immune cells. ETs are extracellular
72 structures composed of chromatin and granule proteins that trap and kill
73 microorganisms (10). ET formation in PMNs is triggered by many pathogen and danger
74 associated molecular patterns (microorganisms, histones, immune complexes,
75 HMGB1), exogenous compounds (PMA, zymosan, calcium and potassium ionophores),
76 platelets, antibodies and inflammatory cytokines, such as IL-8 and TNF (11, 12). MΦ
77 have been reported to produce ETs in response to different pathogens such as
78 *Staphylococcus aureus* (13), *Mycobacterium tuberculosis* (Mtb) (14) and the parasite
79 *Besnoitia besnoiti* (15). However, this antimicrobial mechanism does not seem to be
80 dominant in MΦ, and it seems that this cell type would be more prone to clearing ETs
81 produced by PMNs and resulting apoptotic PMN than to producing ETs (16).
82
83 Indeed, several studies have described the cooperation of PMN and MΦ to remove
84 pathogens at the inflammation site (17, 18). PMN are proposed as key mediators,
85 increasing the MΦ' killing capacity through phagocytosis of apoptotic PMN, in a
86 process called efferocytosis, which can potentially result in cross-presentation. This
87 mechanism provides MΦ with antimicrobial molecules that are lacking in the mature
88 MΦ and that aid in the killing of intracellular pathogens (18, 19). PMN also transfer
89 ingested intracellular pathogens to MΦ during efferocytosis (18).
90
91 In addition to PMN and MΦ, the pro-inflammatory cytokines IL-1β and IL-8 are also
92 essential components in the formation of granulomas in mycobacterial infections. IL-1β
93 is a key mediator of the inflammatory response produced by cells of the innate immune
94 system that further attracts MΦ (20) and leads to a general activation of the immune
95 system. Furthermore, IL-1β seems to play an important role in protecting mice against
96 experimental Map infection (21). Although essential for resistance to infection, IL-1β
97 also exacerbates damage during chronic diseases and acute tissue injuries, and has been
98 suggested that it may be used by Map to attract MΦ to the site of infection, thus

ensuring its survival and dissemination (22). IL-8 release by human PMN is important in leukocyte recruitment, granuloma formation, and respiratory burst in response to Mtb. IL-8 can bind Mtb improving the phagocyte's ability to ingest and kill the bacterium (23) and it is considered as a major ET inducer for PMN (24).

There is little research on the protective role of PMN in Map infection in cattle. PMN isolated from cattle infected sub-clinically with Map showed diminished migratory properties when stimulated *in vitro* compared to PMN of healthy cattle (25), suggesting that Map infection potentially undermines PMN functionality. Indeed, PMN seem to be absent compared to healthy tissue in cows with clinical PTB(26). In the last years, several transcriptome studies have supported the idea that Map infection causes an impairment of PMN recruitment (27, 28) and the downregulation of antimicrobial peptide production by PMN, such as those of beta-defensins (29) and cathelicidins (28). A recent study has shown that cathelicidin LL-37 produced by human PMN restricts Mtb growth (30) and facilitates Map clearance in murine MΦ by suppressing the production of tissue-damaging inflammatory cytokines such as IL-8, TNF and IFN-γ (31). Taken together, these studies suggest that PMN may play a more important role than previously thought during the early stages of Map infection and further studying this immune cell type may provide further evidence for potential novel intervention strategies.

In order to gain knowledge on PMN and Map interaction, we studied the individual and cooperative interaction of bovine monocyte-derived MΦ (MDM) and PMNs, either alone or in co-cultures with Map and BCG, evaluating ET formation, bacterial killing and pro-inflammatory cytokine (IL-1β and IL-8) levels. This is the first study to investigate ET release in response to Map exposure.

Materials and Methods

Animals

Blood samples were drawn from a healthy Holstein Friesian herd in a commercial dairy farm located in the Basque Country. The herd is enrolled in the national bTB eradication program. Animals on the farm tested negative for bTb by skin-test over the last five years. All animals used in this study (n=4) tested negative for Map shedding in

faeces, as confirmed by PCR, and were negative for Map-specific antibodies to Map, as determined by PPA-3 ELISA.

Animals used in this study were submitted only to procedures that according to European (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Chapter 1, Article 1, Section 5, paragraphs b and f) and Spanish (Real Decreto 53/2013, de 1 de febrero, por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia, Article 2, Section 5, Paragraphs b and f) legislation on experimental animals are exempt from its application. The animals, belonging to a registered commercial farm supervised by the local livestock authority (Servicio de Ganadería de la Diputación Foral de Bizkaia) were submitted only to the introduction of a needle in accordance with good veterinary practice and were not killed in relationship with this study.

Bovine peripheral blood monocyte isolation and generation of macrophages

Blood was taken from the jugular vein using a 16G x 11/2 hypodermic needle into a blood-collection bag containing 63 mL of citrate phosphate dextrose adenine (CPDA; TerumoBCT Teruflex) for a total capacity of 450 mL, which was used for monocyte isolation. One month later, 40 mL of blood from the same cows were collected with a 18G hypodermic needle into a 50 mL tube containing 7 mL of CPDA for PMN isolation. Blood samples were processed within two hours of the extraction.

Peripheral blood mononuclear cells (PBMCs) were separated using Histopaque 1077® and monocytes were selected using mouse anti-human CD14-coupled microbeads and MS columns (Miltenyi) following the manufacturer's recommendations. Greater than 90% of purity of monocytes was achieved as determined by flow cytometry using a mouse anti-bovine CD14 FITC-labelled antibody (BioRad).

For the production of MDM, monocytes were plated at $1x10^5$ cells/well for co-cultures and at $2x10^5$ cells/well for MDM cultures in wells of 96-well plates for fluorimetric assays to quantify ET release. For immunofluorescence microscopy, monocytes were plated at $1x10^5$ cells/well for co-cultures and at $2x10^5$ cells/well for MDM cultures on 16-well chamber slides (178599PK Thermo Scientific). For contact independent cultures, monocytes were plated at $5x10^5$ cells/well for co-cultures and at $1x10^6$

cells/well in wells of 24-well plates. During maturation to MΦ, monocytes were cultured in RPMI 1640 media without phenol red (Gibco 11835030), supplemented with 2mM L-glutamine, 10% foetal bovine serum (FBS), 1% penicillin-streptomycin and 40 ng/mL recombinant bovine M-CSF (Kingfisher Biotech, Inc. RP1353B) at 37°C and 5% CO2. The media was replaced every three days and prior to the beginning of the mycobacterial infection assays. After 7 days of culture, MΦ morphology was confirmed by light microscopy (increased size, increased adherence, cytoplasmic granularity and presence of pseudopods).

Bovine neutrophil isolation

A week after PBMC isolation, 25 mL of blood were drawn from the same cows by venepuncture and mixed with 25 mL of PBS supplemented with 2% FBS. 25 mL of the mixture were layered on top of 15 mL of Histopaque® 1077 (Sigma) and centrifuged at 1200 x g for 45 min at RT with no brakes. PMNs were located at the lower end of the tube and were subjected to two flash hypotonic lysis using 25 mL of sterile distilled water during 20-30 sec, followed by 2.5 mL of a 10% NaCl solution and topped up with PBS-2% FBS, in order to eliminate remaining red blood cells. PMNs were then resuspended in RPMI 1640 with glutamine and without phenol red and counted. Over 90% purity was achieved as determined by FSC and SSC gating and visualization of characteristic nuclei after DAPI staining under a fluorescence microscope.

Bacterial strains and culture

Map strains K10, Map K10-GFP (kindly provided by Dr. Jeroen de Buck, University of Calgary, (32)), *M.bovis* BCG Danish strain 1331 and *M. bovis* BCG Danish strain 1331-GFP (32) were used in the assays. All strains were grown to exponential phase at 37°C under aerobic conditions. Map-GFP and BCG-GFP were cultured for three weeks on Middlebrook 7H9 OADC with Kanamycin (25µg/mL) to select for GFP-plasmid carriers and supplementation with Mycobactin J was added for Map. Map strain K10 and BCG Danish strain 1331 were used for ET isolation and quantification and were cultured in the same medium without kanamycin. Cultures were adjusted after reaching an OD of 0.7 ($3x10^8$ bacteria/mL) and colony forming units (CFU) were confirmed on agar after using 10-fold serial dilutions of bacterial suspensions. In experiments comparing the response to live and killed bacteria, heat-inactivation was performed by

heating bacteria for 30 min at 85ºC. Inactivation of bacteria was confirmed by plating on agar plates.

Cell cultures and in vitro infection

A schematic representation of the different cell culture types is shown on Figure 1. PMNs and MDMs isolated from the same cow were cultured separately or co-cultured together in the same well (CC). In order to study cellular changes following paracrine signalling in the absence of cell-to-cell contact between PMNs and MDMs and also to determine ET formation, a transwell co-culture system was also employed. PMNs were seeded in the transwell insert (pore diameter 0.4 µm; Thermo Fisher Scientific 140620) to generate insert PMNs (TW-PMNs), whereas MDMs were seeded into the lower well (TW-MDM). ET release quantification and mycobacteria killing were performed on 24 and 96 well plates. ET visualization was performed on 16-well chamber slides (Nunc Labtek).

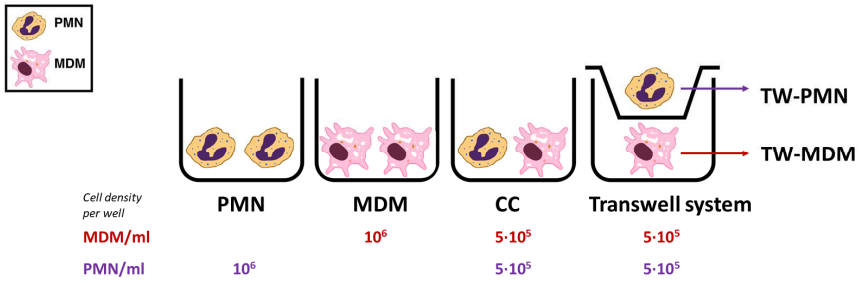


Figure 1. Cell culture types and conditions.

Once cell cultures were set, these were stimulated with live or heat-inactivated Map K10-GFP, *M. bovis* BCG -GFP, Map K10, *M. bovis* BCG at either a MOI of 1 (ET release) or a MOI of 5 (survival assay), zymosan (1mg/mL; Sigma Z4250), or left untreated (negative control). The original bacterial strains with no GFP were used for the isolation and quantification of NETs in the fluorometric assay to avoid GFP fluorescence interference. Cultures were incubated at 37°C and 5% CO_2 for 4h for ET release evaluation and for 24h for bacteria killing and cytokine levels.

Isolation and quantification of ETs

ET release was assayed as described by Köckritz-Blickwede et al (33) with some modifications. Briefly, ETs generated by cells were digested with 500 mU/mL of

micrococcal nuclease (10107921001 Roche) for 10 mins at 37°C 5% CO2. The nuclease activity was stopped by the addition of 5 mM EDTA; thereafter, culture supernatants were collected and stored at 4°C overnight. Total DNA was extracted from naive cells with DNeasy® blood & tissue kit (Qiagen) following the manufacturer's instructions. Extracted DNA was solubilized in TE buffer. Both ETs and genomic DNA was quantified using Quant-iT™ PicoGreen™ assay (Thermofisher) according to the manufacturer´s instructions. Plates were read in a fluorescence microplate reader (Synery HTX, Biotek) with filter settings at 488 nm excitation and 520 nm emission. The percentage of DNA released as ET-DNA was calculated by dividing the amount of isolated ET-DNA by the total amount of genomic DNA.

Visualization and quantification of ETs by immunofluorescence microscopy

To assess histone staining as a marker for ET formation, cells were seeded into 16-well chamber slides for subsequent antibody staining and microscopy according to Conejeros et al (34). After 4 h of incubation with either Map-GFP, BCG-GFP or zymosan, cells were fixed for 15 min with 4 % formaldehyde. Cells were washed twice with PBS and kept in PBS at 4°C until staining was performed. Subsequently, cells were permeabilized for 15 min with PBS 0.1% Triton X-100, blocked with 1% goat serum containing 0.05% Tween 20 and 3% BSA-PBS. The immunofluorescence staining was performed overnight at 4°C using a mouse anti-pan-histone antibody (Merk MAB3422; diluted 1:200). Thereafter, cells were washed three-times with PBS and incubated with anti-mouse Alexa Fluor 594 labelled antibody (Invitrogen A-1105, diluted 1:500) for 30 min at RT. Following further three washes with PBS, wells were detached from the slides and mounting medium containing DAPI was added before coverslips were added. Preparations were let dry and observed at 400x magnification on a DMi8 fluorescence inverted microscope (LEICA®). Pictures were taken using a DFC3000 G camera coupled to the microscope.

Quantification was performed by taking snapshots of five fields containing visible ETs with histone staining. Analysis was performed using the Image J software package. Snapshots in 8-bit format were analysed using the following pipeline. Threshold was adjusted depending on the micrograph, being 4 for low level and 255 for high level. The percentage of picture area occupied by ETs plus nuclei (total DNA) was measured by adjusting the settings for fluorescent particle size: 0-infinity and circularity: 0-1. The

percentage of picture area occupied by nuclei were analysed adjusting the settings for particle size: 200-5000 and circularity: 0.3-1. ET percentage was calculated as total DNA percentage minus nuclei percentage. In the case of cultures that did not contain ETs, nuclei were measured, and ET release was considered zero.

Mycobacteria killing assay

PMNs, MDMs and co-cultures inoculated with BCG -GFP and Map-GFP were prepared. At time 0 (4ºC control) and 24 h, the supernatants were removed and centrifuged to pellet non-internalized mycobacteria. Supernatants were stored for cytokine analysis. Wells were washed twice with PBS to remove the remaining non-internalized bacteria. Bacterial pellets and washes of remaining bacteria were centrifuged together, and the final pellets were resuspended in 100 µL of filtered PBS. Adherent cells were lysed by vigorous pipetting with 0.5 mL of 0.1% Triton X-100 (Sigma-Aldrich) in sterile water for 10 min at RT.

Two serial 10-fold dilutions of each lysed sample and supernatant were performed in a total volume of 1 mL using filtered PBS. 200 µl of each dilution was inoculated in duplicate in Middlebrook 7H9 OADC-Kanamycin (25µg/mL) agar plates, supplemented with mycobactin J in the case of Map cultures. Seeded agar plates were allowed to dry at RT until humidity was no longer visible. Plates were sealed with tape to avoid desiccation and incubated at 37 ± 1 °C for 6 weeks. CFU were counted and 0h (4ºC control) CFU were considered total inoculated bacteria, 24h CFU supernatant bacteria were considered non-internalized non-killed and 24h CFU cell bacteria were considered internalized and non-killed bacteria. Killed bacteria were estimated as total inoculated bacteria minus (24h CFU supernatant bacteria plus 24h CFU cell bacteria). CFU counts were multiplied by the inverse dilution factor and by the seeded volume. The mean of duplicate plates was calculated and the group mean for each culture type was calculated. Supernatants' fraction and cell-lysis' fractions were divided by the 100 % survival control to calculate the survival percentage of each portion and the killing rate.

Cytokine detection assay

Commercially available direct ELISA kits were performed to determine bovine IL-1β (ESS0027 Invitrogen®) and IL-8 (3114-1A-6 MABTECH) release in 24h culture supernatants using the provided manufacturer's instructions. IL-1β ELISA is based on streptavidin-HRP and IL-8 on streptavidin-ALP. Absorbances were measured using an

292 automated ELISA plate reader (Multiskan EX®, Thermo Lab Systems, Finland) and in
293 all cases standard curves were used to determine each cytokine amount in the
294 supernatant samples.
295
296 **Statistics**
297 Normality of data distribution and homoscedasticity were tested using Shapiro-Wilk
298 and Bartlett´s test respectively. All data are presented as the mean +/- standard deviation
299 (SD) of four animals in triplicates for ET release by fluorimetry and in duplicates for
300 bacterial killing and cytokine release. ET% differences between culture type's means
301 were calculated using pairwise comparisons using t tests with pooled SD for all the
302 stimuli except for unstimulated and zymosan stimulated cultures, where
303 homoscedasticity could not be assumed, and comparisons were calculated using non-
304 pooled SD test.
305
306 The total inoculated CFU at 0 h was considered 100% survival. The survival at 24 h was
307 calculated as the sum of internalized mycobacteria CFU and supernatant mycobacteria
308 CFU. The killing rate was calculated as the difference between the CFU at 0h and the
309 survival at 24 h expressed as a percentage. Killing rate means were compared through
310 multiple pairwise comparisons using t tests with non-pooled SD with BH correction. In
311 co-cultures, expected killing rate for each mycobacterial strain was calculated by
312 dividing each individual culture (PMN, MDM) killing rate by two and adding both
313 obtained values.
314
315 Differences in IL-1β levels were compared employing the Kruskal-Wallis test with the
316 post-hoc Dunn's multiple comparison test. IL-8 level differences were calculated
317 through multiple pairwise comparisons using t tests with pooled SD.
318
319 Pearson ($\rho_{x,y}$) and Spearman (ρ) coefficients were calculated for correlation between
320 variables to detect linear and non-linear correlations and in each case the best fitting
321 coefficient value was chosen.
322
323 All the statistical tests were performed using R studio desktop (version 1.2.5033;
324 (RStudio Team, 2019. RStudio: Integrated Development for R. RStudio, Inc., Boston,

MA URL http://www.rstudio.com/.) A p-value <0.05 was considered statistically significant.

Results

PMNs release extracellular traps against both mycobacteria killed and alive in contrast to MDMs

PMNs and MDMs were cultured either separately or together to study ET release as a response to Map and BCG exposure. In order to assess whether this mechanism would rely on live bacteria or whether heat-killed bacteria would be capable of inducing similar effects, all culture cell types were incubated with both live and heat-inactivated Map and BCG.

Fluorometric analysis revealed that both cell types cultured individually extruded similar detectable DNA levels under unspecific stimulation with zymosan, whereas in co-cultures, cells released lower ET levels (Figure 2). ET release levels against mycobacteria were lower and similar for all culture cell types when challenged with live bacteria. In contrast, PMNs showed higher ET release against killed mycobacteria compared to MDMs (K-BCG, p=0.03; K-Map, p=0.065) and to CC (K-BCG, p=0.04; K-Map, p=0.043).

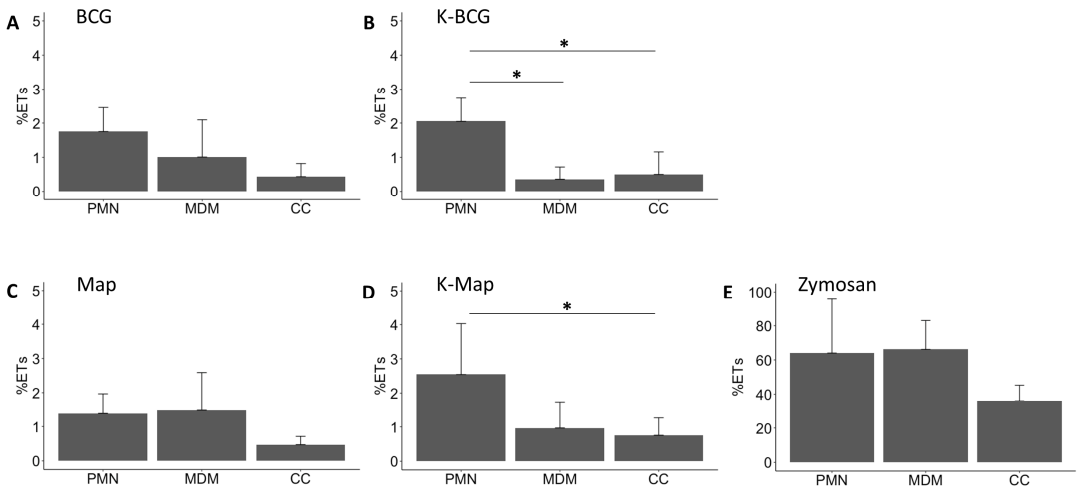


Figure 2. ET release quantified by fluorimetry by PMNs, MDMs and CCs stimulated with BCG, K-BCG, Map, K-Map and zymosan. Data are composed of combined values obtained of all cows (n=4), with samples run in triplicates. * depicts a p value of p < 0.05.

PMNs and MDMs alone or in direct co-cultures exert different effector mechanisms against BCG and Map

Although fluorometric quantification analysis revealed significant differences in ET release among cell culture types, further analysis of images was performed to discriminate between DNA from ET release and DNA liberated by other means. Image analysis quantification of ETs showed that PMNs liberated significantly higher levels of ETs (6.52% - 7.23%) compared to CCs (1.13% - 2.03%) and MDMs (0%) when stimulated with both mycobacteria (Figure 3 graphs). Significant differences were not observed between cells incubated with killed or live mycobacteria. As seen before, MDMs produced ET-like structures only when stimulated with zymosan. MDMs and PMNs, cultured either separately or in direct contact and stimulated with zymosan lost normal nuclei structure, ending in nuclear DNA extrusion (Figure 3 E). However, co-cultures seemed to possess fewer ETs, which could be due to MDMs phagocytosing liberated ETs by PMNs or because direct contact of both cell types inhibits ET release.

In case of ETs being produced by PMN, anti-histone immunolabelling, shown in red, was not always associated with DAPI-stained nuclei but mainly within the extruded ETs (Figure 3A PMNs and 3C PMNs and CC). MDMs showed internalized mycobacteria but did not show ET release (Figure 3A and 3C MDMs). Furthermore, MDM nuclei were always stained with the anti-histone antibody and DAPI when cultured in the presence of BCG or Map live or dead (Figure 3A, 3B, 3C and 3D MDMs). In addition, MDMs showed less mycobacterial internalization when they were in co-culture, whereas the opposite was observed with PMNs (Figure 3A and 3C CCs). However, in Map stimulated CCs, PMNs nuclei were not always labelled by the anti-histone antibody, and many PMNs showed condensed nuclei, potentially indicative of apoptosis (Figure 3C CC). In CC, ETs were rarely detected and MDMs presented a red punctate pattern in the cytoplasm (Figure 3A and 3C CCs) that could be phagocytosed histones liberated by PMNs or by themselves.

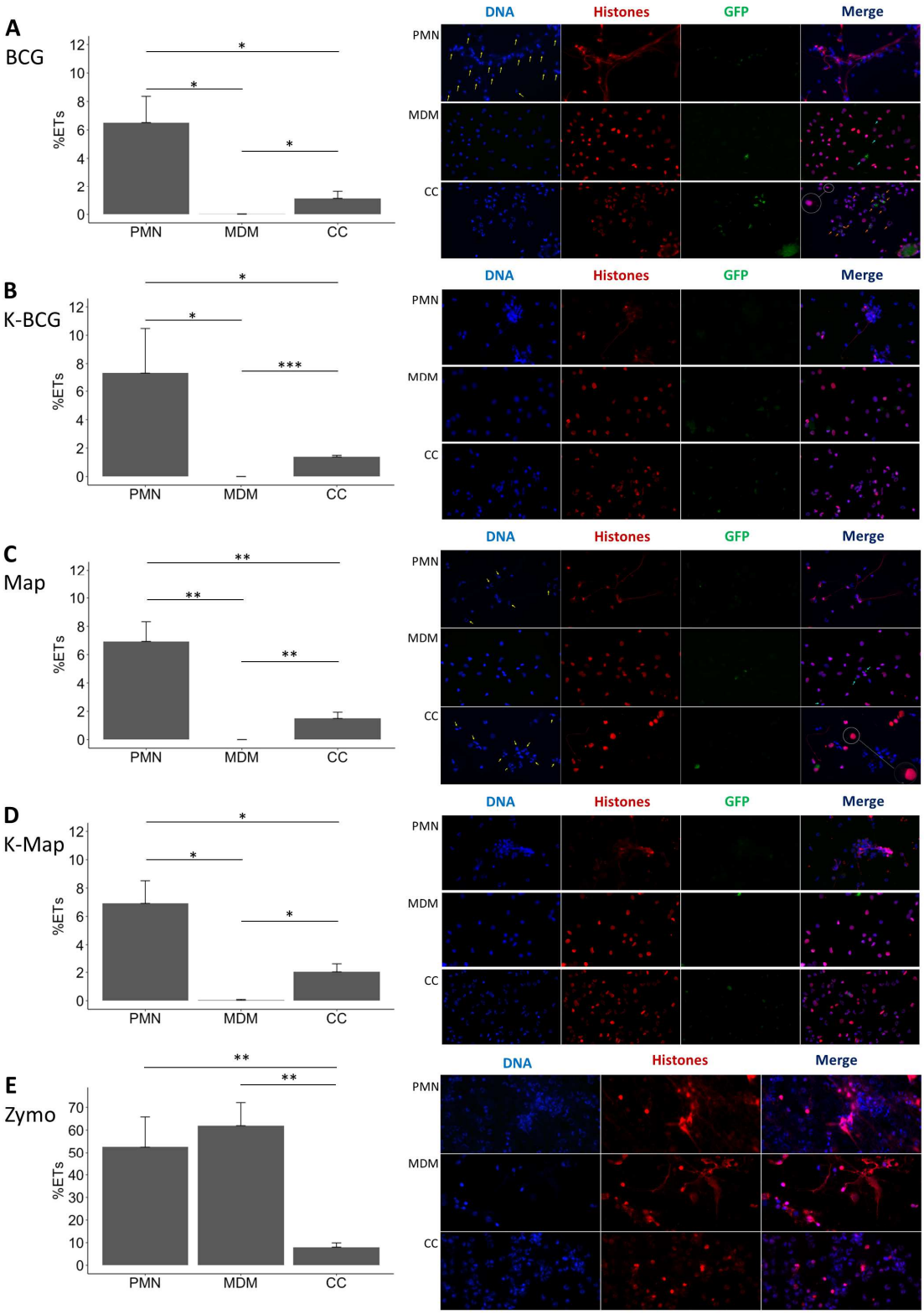


Figure 3. ET release quantified by immunofluorescence microscopy image analysis and micrographs (40x) of A) BCG, B) K-BCG, C) Map, D) K-Map and E) zymosan stimulated PMNs, MDMs and CCs. DAPI stained DNA in blue, histones in red and BCG-GFP or Map-GFP in green. Yellow arrows indicate nuclei stained with DAPI, but not immunolabeled for histones. Light-blue arrows indicate MDM phagocytosis. Orange arrows indicate PMN phagocytosis of BCG. A white circle has an augmented

image showing a red punctate pattern in MDMs. Data composed of combined values obtained of all cows (n=4), with samples run in duplicates and 5 fields quantified per micrograph. (* $p<0.05$; ** $p<0.01$;*** $p<0.001$)

PMNs separated by transwell from MDMs release higher levels of ET in response to mycobacteria

Having established that both mycobacterial species induce ET formation in PMNs and less in MDMs, we next assessed if co-culturing both cell types resulted in an additive effect. Both cell types were cultured either in direct contact or separated by the transwell membrane in order to study if ET release was influenced by paracrine signalling.

In general, ETosis by PMNs alone (Figure 2) was lower compared to that seen in TW-PMN (Figure 4) for all stimuli tested, potentially indicating that MDMs stimulate ET release by PMNs in a paracrine manner. Furthermore, direct cell contact induced the lowest ET release compared to the rest of the culture types and ET release was highest in TW-PMNs compared to all other culture types, independent whether cells were stimulated with either mycobacteria both, inactivated and alive ($p<0.001$). ETosis by TW-PMN being higher in response to Map compared to BCG ($p=0.015$).

The ET release in zymosan-stimulated cultures was relatively homogenous in the transwell system culture conditions (Figure 4E). Interestingly, TW-PMNs and TW-MDMs showed similar ET release levels ranging from 60-70% upon stimulation with zymosan but ET formation was reduced to 36.07+/-9.16 % when PMN and MDM were cultured with direct contact in co-cultures (CC), showing significant differences with TW-PMN (69.96+/-3.34 %) ($p=0.028$).

TW-MDMs showed similar ET release values for both alive and killed Map (Figure 4), whereas TW-PMNs showed a higher ET release when stimulated with live Map (Figure 4C). Statistical analysis revealed that TW-PMNs had higher ETosis values compared to TW-MDMs when stimulated with live Map ($p=0.022$) and K-Map ($p= 0.029$), indicating that PMNs are more prone to ET release in the presence of mycobacteria and paracrine stimulation by MDMs.

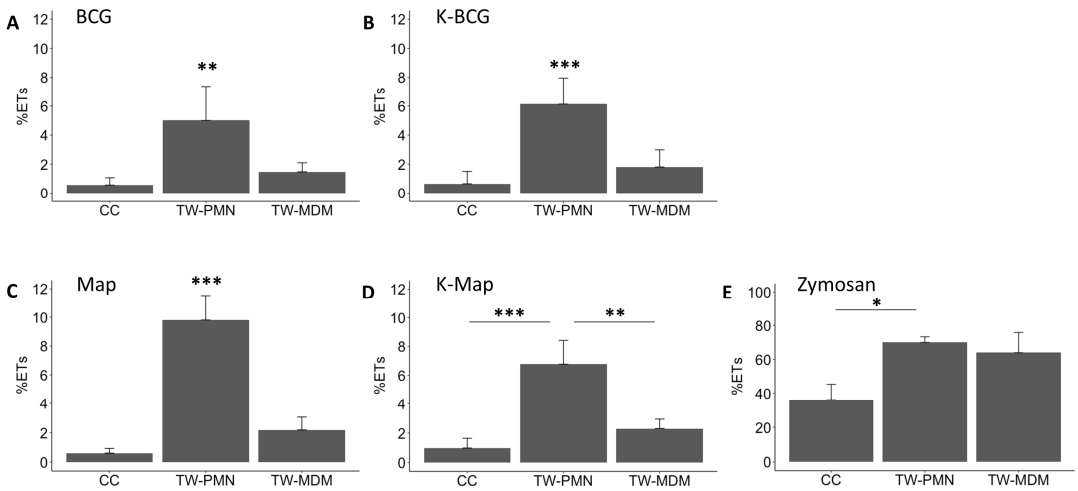


Figure 4. ET release by different culture types CC (in contact co-cultures), TW-PMN (PMNs in transwell co-culture), TW-MDM (MDMs in transwell co-culture) stimulated with K-BCG, BCG, K-Map, Map and zymosan. Data are composed of combined values obtained of all cows (n=4), with samples run in duplicates**.** (* $p<0.05$; ** $p<0.01$;*** $p<0.001$)

PMNs show similar killing levels of Map and BCG, whilst MDMs are more effective at killing BCG

After demonstrating that ET release can be triggered by mycobacteria after 4 hours, we next assessed whether ET formation at an early time point may be beneficial in terms of bacterial clearance. For this purpose, the killing capacity of PMNs, MDMs and co-cultures against both mycobacteria after 24 h was quantified. Total inoculated CFU (0 h) and CFU at 24h for all cultures are shown in Figures 5A and 5C. MDMs and CCs were more effective at killing BCG compared to PMNs alone, whereas PMNs were more effective at killing Map compared to MDMs. Distribution of live and killed BCG and Map in culture cells and supernatant is shown in Figure 5B and Figure 5D.

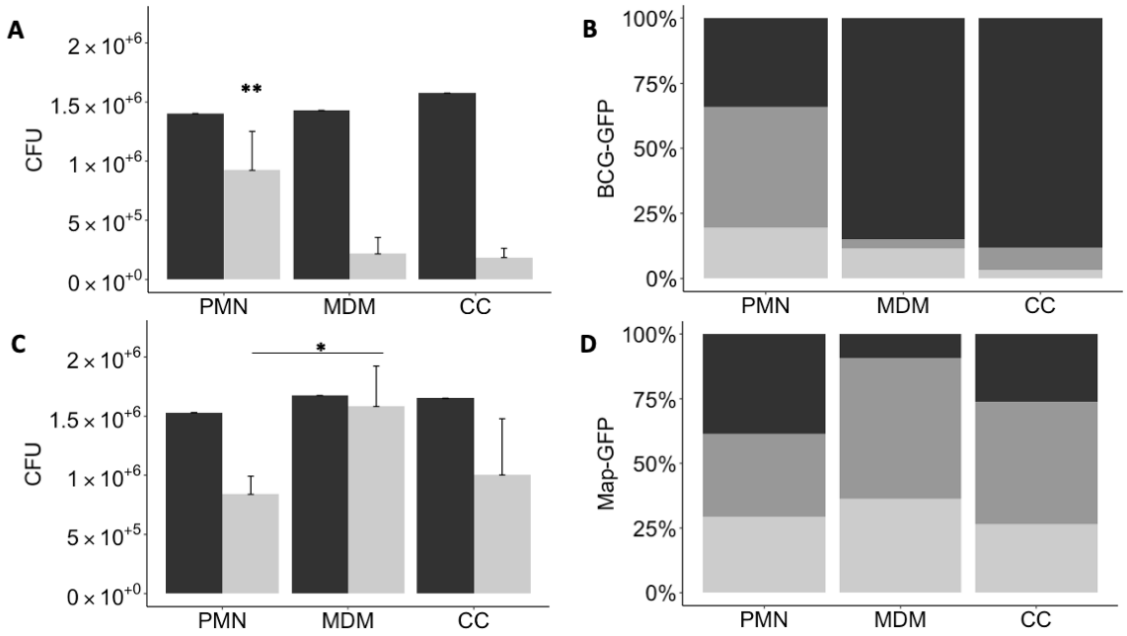


Figure 5. Mycobacterial killing assay results. Distribution of mycobacteria (A) total CFUs of BCG-GFP (B) % BCG-GFP (C) total CFUs Map-GFP (D) % Map-GFP in 24h cultures of PMNs, MDMs and in direct contact co-cultures (CC). In (A) and (C) in black the 0h control and in grey 24h assay. In (B) and (D) in black the fraction of killed bacteria, dark-grey alive bacteria non-internalized from the supernatant and light grey the proportion of internalized bacteria that survived inside the adherent cells. Data composed of combined values obtained of all cows (n=4), with samples run in duplicate (* p=<0.05, **, p=<0.01).

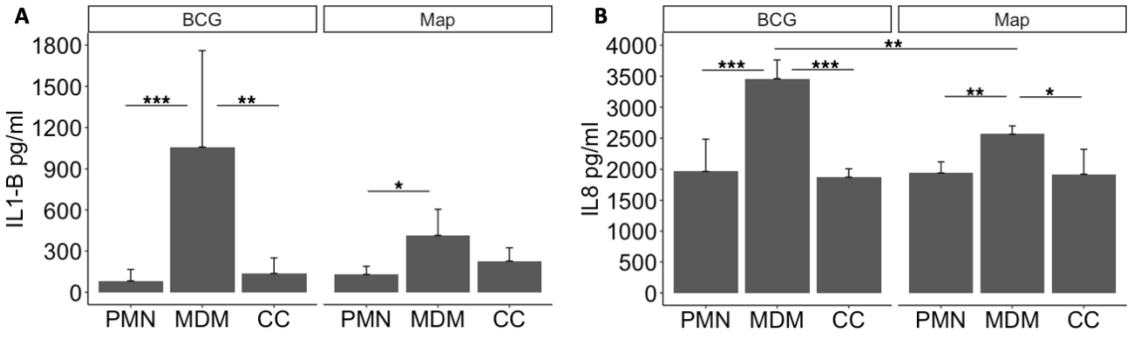
Statistical analysis revealed significant differences between MDMs, PMNs and CCs showing higher killing of BCG in MDM cultures compared to PMNs (84.85% vs 34.15%, p=0.0016), and higher Map killing in PMN cultures than in MDMs (38.36% vs 9.14%, p=0.045). PMN culture killing rate against BCG was lower than in co-cultures (34.14% vs 88.19%; p=0.001), whereas MDM culture killing rate against Map was lower compared to co-cultures (9.14% vs 26.10%; p=0.065), although it was not statistically significant. Considering that CCs include half of the amount of each cell type, and that each cell type shows a certain killing capacity in individual culture, results indicate that co-cultures infected with BCG showed improved killing results (expected killing: 59.49% vs observed killing: 88.19%). In contrast, this synergic effect was not evident in co-cultures infected with Map, showing only 2.35% of improvement (expected killing: 76.24% vs observed killing: 73.89%).

When bacterial survival inside adherent cells of each cell-culture type was compared (Figure 6B and 6D), significant differences were not observed for either BCG or Map. However, non-internalized BCG survival in PMN cultures (46.23%) was higher than in

455 MDM cultures (3.44%, p=0.021) and in co-cultures (8.65%, p=0.021), whereas non-
456 internalized Map survival in PMN cultures was lower than in MDM cultures (54.46%
457 vs 32.17%, p=0.03). Map-GFP survival inside adherent cells was higher than BCG-GFP
458 as expected.

459 **MDMs alone release higher levels of IL-1β and IL-8 compared to PMNs and alone**
460 **or co-cultured with PMNs in response to both mycobacteria**

461 Having assessed the killing capacity of both cell types to different mycobacterial strains,
462 we next investigated whether their cytokine response follows a similar pattern.
463 Considering that IL-8 is a major ET inducer in PMNs (23) and IL-1β is a key mediator
464 of the inflammatory response that attracts MΦ to the infection site (19), we also
465 analysed the levels of these cytokines. IL-1β release after 24h showed significant
466 differences between the culture types against both mycobacteria (Figure 6A). MDM
467 cultures released higher concentrations of IL-1β in response to infection with BCG in
468 comparison to PMN (p<0.001) and CC (p=0.009) as well as in response to infection
469 with Map in comparison with PMN (p=0.035).
470
471 IL-8 levels after 24 hours are shown in Figure 6B. Similar as seen for IL-1β, MDMs
472 released more IL-8 into the supernatant compared to co-cultures (BCG; p< 0.001, Map;
473 p=0.013) or PMNs alone (BCG; p=0.001, Map; p= 0.005). As before, MDMs
474 challenged with BCG produced significantly more IL-8 than challenge with Map
475 (p=0.011).



476

477 **Figure 6.** (A) IL-1β and (B) IL-8 release of PMN, MDM and CC stimulated with BCG
478 and Map after 24h. Data composed of combined values obtained of all cows (n=4), with
479 samples run in duplicate (* p<0.05, **; p<0.01; *** p<0.001).

480

Correlation analysis of cytokine production and bacteria killing rates suggest different mechanism interplay between MDMs and PMNs against BCG and Map.

Correlation analyses were performed between cytokine levels, and mycobacteria killing rates to evaluate the strength of relationship between both parameters (Table 1). The correlation study was performed including the data from all the culture types together and then subdivided by culture type and stimuli. IL-1β and IL-8 levels were positively correlated at 24h including all culture types in the analysis indicating that both cytokines are secreted in the same culture conditions in our assay. This correlation improved when eliminating the CCs from the analysis suggesting that cytokine release follows a different pattern in CCs. BCG-stimulated cultures showed a positive correlation between IL-1β and killing rate, which increased when eliminating CCs from the analysis. On the other hand, Map-stimulated cultures showed a negative correlation between IL-1β and killing rates, and in this case power was increased when analysing PMNs alone. IL-8 and killing capacity were positively correlated in MDM cultures. IL-8 and killing rates were negatively correlated in Map-stimulated cultures.

Discussion

Map is the etiological agent of PTB, a chronic enteritis that affects livestock production worldwide. Furthermore, Map provides a potential zoonotic risk and is discussed to be associated with the occurrence of Crohn's Disease in humans (35, 36). Research into the pathogenesis of PTB on the cellular level has mainly focused on the interaction of Map with MΦ since these have been considered to be the main immune cell subset invaded by this intracellular pathogen. Less attention has been given to the role PMN play in the pathogenesis of PTB, although these are present at sites showing pathological alterations during early stages of Map infection (5). In the present work, we have investigated some aspects of the interaction of bovine PMN and MDM *in vitro* with Map and BCG, a better studied mycobacterium. Furthermore, we assessed whether cooperation of both phagocytic cells is advantageous for the elimination of these mycobacteria, analysing ET release, pro-inflammatory cytokine levels and killing of mycobacteria. To the best of our knowledge, this is the first study describing ET release against Map.

512 Bovine PMNs and MDMs were cultured separately and together. PMNs showed higher
513 ET release against killed mycobacteria in comparison to MDMs indicating that
514 inactivated Map and BCG can trigger this mechanism in PMNs. Direct contact co-
515 cultures showed less ET release compared to transwell system culture PMNs (TW-
516 PMNs) and MDMs (TW-MDMs). Indeed, this was observed for all stimuli tested;
517 zymosan and both mycobacteria, independent whether these were alive or inacivated.
518 This generalized lower ET detection in direct co-cultures could be due to ET removal by
519 efferocytosis, a process in which apoptotic cells (PMNs and/or MDMs) are removed by
520 phagocytic cells (MDMs) or to a more powerful paracrine signalling activation of
521 PMNs for ETosis by MDMs in the absence of cell-to-cell contact.
522
523 TW-PMNs were the cell culture type showing significantly higher ET release
524 challenged with both, inactivated or live mycobacteria, whereas TW-MDMs liberated
525 low levels of ETs, comparable to CC. In fact, ET release by TW-PMNs was higher in
526 presence of live Map. These results suggest that mycobacteria trigger PMN ETosis, and
527 that soluble factors secreted by MDMs, when cell-to-cell contact is not possible, further
528 drive PMNs in this direction, particularly when Map is alive. However, in cell-to-cell
529 contact interactions ET release would not be the principal mechanism exerted by PMNs.
530 Actually, PMNs in co-culture (CC) present more internalized BCG bacilli and less
531 detectable ETs. In contrast, in Map stimulated CCs, PMNs appear to have condensed
532 nuclei indicative of apoptosis and no detectable histones, rarely detectable ETs and few
533 internalized Map bacilli, whereas MDMs have histones in their cytoplasm.
534
535 Histones can be actively secreted into the extracellular space by activated inflammatory
536 cells (37) and by ET release (38) or are derived more passively by apoptotic and
537 necrotic cells (37). Inhibition of efferocytosis by extracellular histones has been
538 reported in both *in vitro* and *in vivo* conditions (39). In our experimental conditions, we
539 believe that in Map stimulated CCs, activated or apoptotic PMNs liberate histones alone
540 or with ETs that are engulfed subsequently by MDMs, inhibiting efferocytosis and for
541 this reason apoptotic PMNs are still present but ETs are not detectable.
542
543 With regards to the differences seen for bacterial killing, these were most pronounced
544 against both mycobacteria in MDM alone. MDM were highly efficient in killing the
545 attenuated BCG strain, but they seemed to be unable to kill Map in the tested

546 conditions. In contrast, PMNs showed a similar killing capacity against both
547 mycobacteria species tested. Interestingly though, when both immune cell types were
548 co-cultured in a 1:1 ratio, the killing capacity was unaltered with regards to BCG, but
549 was similar to that obtained in PMNs for Map. Nevertheless, taking into account that
550 CCs are composed by half of each cell type compared to MDM or PMN cultures, the
551 obtained CC results indicate that a synergistic effect (observed killing > expected
552 killing) in terms of improved killing capacity in BCG-infected co-cultures is observed,
553 whereas in Map-infected co-cultures the effect was additive (observed killing =
554 expected killing).
555
556 Independent of the condition, our results indicate that PMNs alone are effective at
557 killing Map and that their presence at the infection site in initial stages can be
558 potentially beneficial for the resolution of the disease. Indeed, Brown et al. (40) and
559 Jones et al. (41) demonstrated that human PMN are capable of controlling the infection
560 of Mtb *in vitro* and another study showed that murine PMN are able to kill
561 *Mycobacterium avium ex vivo* when activated *in vivo* with G-CSF (42).
562
563 In general, MDMs presented higher release of the assayed cytokines compared to PMNs
564 and CCs against both mycobacteria. Cytokine levels of co-cultures were lower than
565 expected, resulting equivalent to those obtained in cultures of PMNs alone. A study by
566 Sawant and McMurray showed an additive effect of IL-8 and IL-1β release in co-
567 cultures of guinea pig PMN with alveolar MΦ challenged *in vitro* with Mtb (43). It is
568 likely that those results were obtained because their co-cultures harboured PMN and
569 MΦ in a 1:1 ratio, but doubling the total amount of cells, whereas our study halves the
570 amount of each cell type to preserve the total cell amount. In addition, differences on
571 host and mycobacteria species and MDM sources (lung isolated cells in their case and
572 peripheral isolated cells in our case), make a comparison between both studies difficult.
573
574 Although traditionally considered a product predominantly secreted by monocytes and
575 MΦ, PMN, are also capable of producing IL-1β, although at lower levels (44). Our
576 results are consistent with this fact. MDMs alone produced higher IL-1β quantities than
577 PMNs against both mycobacterial species. Anti-inflammatory mediators released from
578 phagocytes act in an autocrine/paracrine manner, suppressing the expression of IL-1β

579 and other inflammatory cytokines (45). This could be the explanation of the observed
580 lower IL-1β levels in CCs. Indeed, the high IL-1β and IL-1α levels described by others
581 in PTB (46) could be reasonably explained by the already described PMN activation
582 and recruitment impairment, (28, 29; 47- 49) and the low numbers of PMN associated
583 to PTB granulomatous lesions (50, 51). Moreover, high levels of these cytokines are
584 proposed as candidates responsible for PTB inflammation process and contributors to
585 the development of the Th17 response during the final stages of the disease (52).
586
587 IL-8 expression has also been described to increase in PTB and monocytes stimulated
588 with Map (53, 54) compared to controls. In our study IL-8 secretion of the same
589 individuals against BCG and Map was compared. The lower IL-8 release of Map versus
590 BCG stimulated MDMs could be a reflection of higher pathogenicity of Map in MDMs.
591 In this line, a study of a Mtb multidrug resistant strain, revealed a novel survival
592 strategy of the mentioned strain inside lung epithelial cells by decreasing cells' release
593 of IL-8, limiting PMN recruitment to the site of infection (55).
594
595 A positive correlation between IL-1β and IL-8 release has been observed in the present
596 experiment. An increase of both cytokines is positively correlated with the killing of
597 BCG, being higher in MDMs. However, Map infection is negatively correlated with
598 killing and the effect is stronger in PMNs. Low IL-8 levels have been associated to poor
599 prognosis in hTB (23) and survival of *Mtb* inside MΦ by altering PMN effector
600 functions (55). In the present experiments, IL-8 levels were high in PMN, MDM and
601 co-cultures, indicating that despite the negative correlation with Map killing, more
602 factors are involved in this interaction. As for IL-1β, previous studies have shown that
603 IL-1 knock-out mice show an increased susceptibility to Mtb infection, developing a
604 higher bacterial burden and mortality (56). With this in mind, we would have expected
605 that the lowest mycobacterial survival rate would be seen in the cultures with the
606 highest levels of IL-1β. Whereas we saw this in the case of BCG, it was not the case of
607 Map-stimulated MDM cultures, indicating once again that more factors are involved. In
608 fact, *in vitro* studies indicate that Map infection promotes a self-destruction state in the
609 epithelium caused by increased IL-1β levels that attract MΦ, thus providing Map with
610 an escape route from destruction (57).
611

Based on the results obtained in our experiments, we hypothesize that bovine PMN may have an important role against Map. PMN trigger inflammatory responses through IL-1β release and start an effective response phagocytosing and immobilizing bacteria with their NETs. As a consequence, MΦ are activated and attracted by IL-1β production to the site of infection. Upon arrival, MΦ would phagocytose neutrophils' ETs and apoptotic rests, which provides them with the antimicrobial compounds produced by neutrophils' after first contact with Map, thus downregulating the inflammatory response leaded by IL-1β, IL-23 and IL-17 expression, ending ultimately in Map destruction. The timing is probably important in this cooperation and if MΦs arrive at the infection site too early, PMN might not have enough time to effectively expand their antimicrobial mechanisms, MΦ will be the dominant cell type attempting Map elimination but end-up being taken over by Map as a Trojan horse. PMN have been postulated as innate effectors of TB resistance due to their highly effective antimicrobial effector mechanisms they can expand during the early stage of Mtb infection (58). Applied to Map, it could be that harbouring a particular number of competent and tightly regulated PMN capable of expanding the correct antimicrobial mechanism at the specific time may be the difference between being resistant to PTB or developing subclinical or clinical disease.

Conclusions

Taking all assayed parameters into consideration, we could conclude that cooperation of MDMs and PMNs may confer advantages through different mechanisms depending on the pathogen. As for BCG, co-cultures show good levels of elimination of the mycobacteria (high killing rates) and lower cytokine levels and ET release than the PMN and MDM cultures, which, could translate to a decreased tissue damage in the living organism, since an excessive inflammatory reaction would not take place. In Map stimulated cultures, co-cultures show slightly worse figures for mycobacteria killing compared to PMNs but improved rates compared to MDMs, also with lower levels of pro-inflammatory cytokines. In BCG, ET release is negatively correlated to bacterial killing, suggesting that killing could be due to phagocytosis, whereas as in Map ET release is positively correlated to bacterial killing, suggesting that probably most killing is due to PMNs ETosis and provision of macrophages with killing capacity molecules in the co-cultures. Further research should involve *in vivo* studies to confirm that PMNs

644 are necessary in the defence against Map and studies clarifying which soluble factors
645 secreted by MDMs drive PMNs towards ET, and which subsets of PMNs are involved
646 in each effector mechanism in order to develop therapeutic agents targeted at improving
647 PMN competence.

648 Conflict of Interest

649 The authors declare that the research was conducted in the absence of any commercial
650 or financial relationships that could be construed as a potential conflict of interest.

651 Author Contributions

652 DW and NE conceived the study. AU carried out the animal sampling. IL, EM, AH, JK,
653 HH and NE performed the laboratory work. IL and NE compiled and analyzed the data.
654 IL and NE collated the results. IL, NE, JA and DW drafted the preliminary manuscript.
655 All authors participated in the review and the editing of the final draft and also read and
656 approved its final version.

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667 References

668 1. Hasonova L, Hasonova L, Pavlik I. Economic impact of paratuberculosis in dairy
669 cattle herds: a review. *Vet Med* (2006) **51**:193–211. doi:10.17221/5539-
670 VETMED
671 2. Bastida F, Juste RA. Paratuberculosis control: A review with a focus on
672 vaccination. *J Immune Based Ther Vaccines* (2011) **9**: doi:10.1186/1476-8518-9-

3. Juste RA, Alonso-Hearn M, Molina E, Geijo M, Vazquez P, Sevilla IA, Garrido JM. Significant reduction in bacterial shedding and improvement in milk production in dairy farms after the use of a new inactivated paratuberculosis vaccine in a field trial. *BMC Res Notes* (2009) **2**:233. doi:10.1186/1756-0500-2-233

4. Reddacliff L, Eppleston J, Windsor P, Whittington R, Jones S. Efficacy of a killed vaccine for the control of paratuberculosis in Australian sheep flocks. *Vet Microbiol* (2006) **115**:77–90. doi:10.1016/j.vetmic.2005.12.021

5. Khare S, Nunes JS, Figueiredo JF, Lawhon SD, Rossetti CA, Gull T, Rice-Ficht AC, Adams LG. Early phase morphological lesions and transcriptional responses of bovine ileum infected with *Mycobacterium avium* subsp. *paratuberculosis*. *Vet Pathol* (2009) **46**:717–728. doi:10.1354/vp.08-VP-0187-G-FL

6. Jimbo S, Suleman M, Maina T, Prysliak T, Mulongo M, Perez-Casal J. Effect of *Mycoplasma bovis* on bovine neutrophils. *Vet Immunol Immunopathol* (2017) **188**:27–33. doi:10.1016/j.vetimm.2017.04.011

7. Cassatella MA, Locati M, Mantovani A. Never underestimate the power of a neutrophil. *Immunity* (2009) **31**:698–700. doi:10.1016/j.immuni.2009.10.003

8. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. Neutrophil Extracellular Traps Kill Bacteria. *Science* (2004) **303**:1532–1535. doi:10.1126/science.1092385

9. Aulik NA, Hellenbrand KM, Czuprynski CJ. *Mannheimia haemolytica* and its leukotoxin cause macrophage extracellular trap formation by bovine macrophages. *Infect Immun* (2012) **80**:1923–1933. doi:10.1128/IAI.06120-11

10. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, Weinrauch Y, Brinkmann V, Zychlinsky A. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* (2007) **176**:231–241. doi:10.1083/jcb.200606027

11. Skendros P, Mitroulis I, Ritis K. Autophagy in neutrophils: From granulopoiesis to neutrophil extracellular traps. *Front Cell Dev Biol* (2018) **6**: doi:10.3389/fcell.2018.00109

12. Shrestha S, Lee JM, Hong CW. Autophagy in neutrophils. *Korean J Physiol Pharmacol* (2020) **24**:1–10. doi:10.4196/kjpp.2020.24.1.1

13. Benati D, Ferro M, Savino MT, Ulivieri C, Schiavo E, Nuccitelli A, Pasini FL, Baldari CT. Opposite effects of simvastatin on the bactericidal and inflammatory

response of macrophages to opsonized *S. aureus*. *J Leukoc Biol* (2010) **87**:433–442. doi:10.1189/jlb.0409273

14. Wong KW, Jacobs WR. *Mycobacterium tuberculosis* exploits human interferon γ to stimulate macrophage extracellular trap formation and necrosis. *J Infect Dis* (2013) **208**:109–119. doi:10.1093/infdis/jit097

15. Muñoz-Caro T, Silva LMR, Ritter C, Taubert A, Hermosilla C. *Besnoitia besnoiti* tachyzoites induce monocyte extracellular trap formation. *Parasitol Res* (2014) **113**:4189–4197. doi:10.1007/s00436-014-4094-3

16. Boe DM, Curtis BJ, Chen MM, Ippolito JA, Kovacs EJ. Extracellular traps and macrophages: new roles for the versatile phagocyte. *J Leukoc Biol* (2015) **97**:1023–1035. doi:10.1189/jlb.4ri1014-521r

17. Silva MT. When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. *J Leukoc Biol* (2010) **87**:93–106. doi:10.1189/jlb.0809549

18. Silva MT, Silva MN, Appelberg R. Neutrophil-macrophage cooperation in the host defence against mycobacterial infections. *Microb Pathog* (1989) **6**:369–380. doi:10.1016/0882-4010(89)90079-X

19. Tan BH, Meinken C, Bastian M, Bruns H, Legaspi A, Ochoa MT, Krutzik SR, Bloom BR, Ganz T, Modlin RL, et al. Macrophages acquire neutrophil granules for antimicrobial activity against intracellular pathogens. *J Immunol* (2008) **180**:664.2-664. doi:10.4049/jimmunol.180.1.664-a

20. Lopez-Castejon G, Brough D. Understanding the mechanism of IL-1β secretion. *Cytokine Growth Factor Rev* (2011) **22**:189–195. doi:10.1016/j.cytogfr.2011.10.001

21. Kenefick KB, Adams JL, Steinberg H, Czuprynski CJ. In vivo administration of a monoclonal antibody against the type I IL-1 receptor inhibits the ability of mice to eliminate *Mycobacterium paratuberculosis*. *J Leukoc Biol* (1994) **55**:719–722. doi:10.1002/jlb.55.6.719

22. Dinarello CA. Anti-inflammatory Agents: Present and Future. *Cell* (2010) **140**:935–950. doi:10.1016/j.cell.2010.02.043

23. Krupa A, Fol M, Dziadek BR, Kepka E, Wojciechowska D, Brzostek A, Torzewska A, Dziadek J, Baughman RP, Griffith D, et al. Binding of CXCL8/IL-8 to *Mycobacterium tuberculosis* modulates the Innate Immune Response.

Mediators Inflamm (2015)**2015:**124762. doi:10.1155/2015/124762

24. Gupta AK, Hasler P, Holzgreve W, Gebhardt S, Hahn S. Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. *Hum Immunol* (2005) **66**:1146–1154. doi:10.1016/j.humimm.2005.11.003

25. Dotta U, Guglielmino R, Cagnasso A, D'Angelo A, Prato S, Bosso M. Effects of subclinical bovine paratuberculosis on in-vitro polymorphonuclear neutrophil migration. *J Comp Pathol* (1999) **121**:399–403. doi:10.1053/jcpa.1999.0033

26. Coussens PM. Model for immune responses to *Mycobacterium avium* subspecies *paratuberculosis* in cattle. *Infect Immun* (2004) **72**:3089–3096. doi:10.1128/IAI.72.6.3089-3096.2004

27. Park H-E, Park H-T, Jung YH, Yoo HS. Gene expression profiles of immune-regulatory genes in whole blood of cattle with a subclinical infection of *Mycobacterium avium* subsp. *paratuberculosis*. *PLoS One* (2018) **13**:e0196502. doi:10.1371/journal.pone.0196502

28. Alonso-Hearn M, Canive M, Blanco-Vazquez C, Torremocha R, Balseiro A, Amado J, Varela-Martinez E, Ramos R, Jugo BM, Casais R. RNA-Seq analysis of ileocecal valve and peripheral blood from Holstein cattle infected with *Mycobacterium avium* subsp. *paratuberculosis* revealed dysregulation of the CXCL8/IL8 signaling pathway. *Sci Rep* (2019) **9**:14845. doi:10.1038/s41598-019-51328-0

29. David J, Barkema HW, Guan LL, De Buck J. Gene-expression profiling of calves 6 and 9 months after inoculation with *Mycobacterium avium* subspecies *paratuberculosis*. *Vet Res* (2014) **45**:96. doi:10.1186/s13567-014-0096-5

30. Martineau AR, Newton SM, Wilkinson KA, Kampmann B, Hall BM, Nawroly N, Packe GE, Davidson RN, Griffiths CJ, Wilkinson RJ. Neutrophil-mediated innate immune resistance to mycobacteria. *J Clin Invest* (2007) **117**:1988–1994. doi:10.1172/JCI31097

31. Cirone KM, Lahiri P, Holani R, Tan YL, Arrazuria R, De Buck J, Barkema HW, Cobo ER. Synthetic cathelicidin LL-37 reduces *Mycobacterium avium* subsp. *paratuberculosis* internalization and pro-inflammatory cytokines in macrophages. *Cell Tissue Res* (2020) **379**:207–217. doi:10.1007/s00441-019-03098-4

32. Parker AE, Bermudez LE. Expression of the green fluorescent protein (GFP) in *Mycobacterium avium* as a tool to study the interaction between Mycobacteria

and host cells. *Microb Pathog* (1997) **22**:193–198. doi:10.1006/mpat.1996.0106

33. Von Köckritz-Blickwede M, Chow O, Ghochani M, Nizet V. Visualization and functional evaluation of phagocyte extracellular traps. *Methods in Microbiology* (2010) **37**:139-160. doi:10.1016/S0580-9517(10)37007-3

34. Conejeros I, Velásquez ZD, Grob D, Zhou E, Salecker H, Hermosilla C, Taubert A. Histone h2a and bovine neutrophil extracellular traps induce damage of *Besnoitia besnoiti*-infected host endothelial cells but fail to affect total parasite proliferation. *Biology (Basel)* (2019) **8**:78. doi:10.3390/biology8040078

35. Behr MA, Kapur V. The evidence for *Mycobacterium paratuberculosis* in Crohn's disease. *Curr Opin Gastroenterol* (2008) **24**:17–21. doi:10.1097/MOG.0b013e3282f1dcc4

36. Juste RA, Elguezabal N, Garrido JM, Pavon A, Geijo M V., Sevilla I, Cabriada J-L, Tejada A, García-Campos F, Casado R, et al. On the Prevalence of *M. avium* Subspecies *paratuberculosis* DNA in the Blood of Healthy Individuals and Patients with Inflammatory Bowel Disease. *PLoS One* (2008) **3**:e2537. doi:10.1371/journal.pone.0002537

37. Xu J, Zhang X, Monestier M, Esmon NL, Esmon CT. Extracellular Histones Are Mediators of Death through TLR2 and TLR4 in Mouse Fatal Liver Injury. *J Immunol* (2011) **187**:2626–2631. doi:10.4049/jimmunol.1003930

38. Papayannopoulos V, Zychlinsky A. NETs: a new strategy for using old weapons. *Trends Immunol* (2009) **30**:513–521. doi:10.1016/j.it.2009.07.011

39. Friggeri A, Banerjee S, Xie N, Cui H, de Freitas A, Zerfaoui M, Dupont H, Abraham E, Liu G. Extracellular histones inhibit efferocytosis. *Mol Med* (2012) **18**:825–833. doi:10.2119/molmed.2012.00005

40. Arthur E. Brown, Timothy J. Holzer, Burton R. Andersen. Capacity of Human Neutrophils to Kill *Mycobacterium tuberculosis. The Journal of Infectious Diseases* (1987) **156**: 985-989. doi: 10.1093/infdis/156.6.985

41. Jones GS, Amirault HJ, Andersen BR. Killing of *Mycobacterium tuberculosis* by neutrophils: A nonoxidative process. *J Infect Dis* (1990) **162**:700–704. doi:10.1093/infdis/162.3.700

42. Bermudez LE, Petrofsky M, Stevens P. Treatment with recombinant granulocyte colony-stimulating factor (Filgrastin(TM)) stimulates neutrophils and tissue macrophages and induces an effective non-specific response against *Mycobacterium avium* in mice. *Immunology* (1998) **94**:297–303.

doi:10.1046/j.1365-2567.1998.00529.x

43. Sawant K V., McMurray DN. Guinea pig neutrophils infected with *Mycobacterium tuberculosis* produce cytokines which activate alveolar macrophages in noncontact cultures. *Infect Immun* (2007) **75**:1870–1877. doi:10.1128/IAI.00858-06

44. Malyak M, Smith MF, Abel AA, Arend WP. Peripheral blood neutrophil production of interleukin-1 receptor antagonist and interleukin-1β. *J Clin Immunol* (1994) **14**:20–30. doi:10.1007/BF01541172

45. Vandivier RW, Henson PM, Douglas IS. Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease. *Chest* (2006)**129**:1673-82. doi: 10.1378/chest.129.6.1673

46. Chiang SK, Sommer S, Aho AD, Kiupel M, Colvin C, Tooker B, Coussens PM. Relationship between *Mycobacterium avium* subspecies *paratuberculosis*, IL-1α, and TRAF1 in primary bovine monocyte-derived macrophages. *Vet Immunol Immunopathol* (2007) **116**:131–144. doi:10.1016/j.vetimm.2007.01.005

47. Marfell BJ, O'Brien R, Griffin JFT. Global gene expression profiling of monocyte-derived macrophages from red deer (*Cervus elaphus*) genotypically resistant or susceptible to *Mycobacterium avium* subspecies *paratuberculosis* infection. *Dev Comp Immunol* (2013) **40**:210–217. doi:10.1016/j.dci.2013.02.004

48. Gossner A, Watkins C, Chianini F, Hopkins J. Pathways and Genes Associated with Immune Dysfunction in Sheep Paratuberculosis. *Sci Rep* (2017) **7**:46695. doi:10.1038/srep46695

49. Ibeagha-Awemu E, Do D, Dudemaine P, Bissonnette N. PSXIV-17 Gene co-expression network analysis identifies important modules and genes for cow's response to *Mycobacterium avium* ssp. *paratuberculosis* infection in the small intestine. *J Anim Sci* (2018) **96**:39. doi:10.1093/jas/sky404.088

50. Krüger C, Köhler H, Liebler-Tenorio EM. Cellular composition of granulomatous lesions in gut-associated lymphoid tissues of goats during the first year after experimental infection with *Mycobacterium avium* subsp. *paratuberculosis*. *Vet Immunol Immunopathol* (2015) **163**:33–45. doi:10.1016/j.vetimm.2014.11.002

51. Lee H, Stabel JR, Kehrli ME. Cytokine gene expression in ileal tissues of cattle infected with *Mycobacterium paratuberculosis*. *Vet Immunol Immunopathol* (2001) **82**:73–85. doi:10.1016/S0165-2427(01)00340-3

52. DeKuiper JL, Coussens PM. Inflammatory th17 responses to infection with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in cattle and their potential role in the development of johne's disease. *Veterinary Immunology and Immunopathology* (2019) **218**:109954. doi:10.25335/7F47-KM75

53. Weiss DJ, Evanson OA, Moritz A, Deng MQ, Abrahamsen MS. Differential responses of bovine macrophages to *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium*. *Infect Immun* (2002) **70**:5556–5561. doi:10.1128/IAI.70.10.5556-5561.2002

54. Coussens PM, Verman N, Coussens MA, Elftman MD, McNulty AM. Cytokine Gene Expression in Peripheral Blood Mononuclear Cells and Tissues of Cattle Infected with *Mycobacterium avium* subsp. *paratuberculosis*: Evidence for An Inherent Proinflammatory Gene Expression Pattern. *Infect Immun* (2004) **72**:1409–1422. doi:10.1128/IAI.72.3.1409-1422.2004

55. Kviatcovsky D, Rivadeneyra L, Balboa L, Yokobori N, López B, Ritacco V, Schattner M, Sasiain MDC, De La Barrera S. *Mycobacterium tuberculosis* Multidrug-Resistant Strain M Induces Low IL-8 and Inhibits TNF- α Secretion by Bronchial Epithelial Cells Altering Neutrophil Effector Functions. *Mediators Inflamm* (2017) **2017**:2810606. doi:10.1155/2017/2810606

56. Mayer-Barber KD, Barber DL, Shenderov K, White SD, Wilson MS, Cheever A, Kugler D, Hieny S, Caspar P, Núñez G, et al. Cutting Edge: Caspase-1 Independent IL-1β Production Is Critical for Host Resistance to *Mycobacterium tuberculosis* and Does Not Require TLR Signaling In Vivo . *J Immunol* (2010) **184**:3326–3330. doi:10.4049/jimmunol.0904189

57. Lamont EA, O'Grady SM, Davis WC, Eckstein T, Sreevatsan S. Infection with *Mycobacterium avium* subsp. *paratuberculosis* results in rapid interleukin-1β release and Macrophage transepithelial migration. *Infect Immun* (2012) **80**:3225–3235. doi:10.1128/IAI.06322-11

58. Kroon EE, Coussens AK, Kinnear C, Orlova M, Möller M, Seeger A, Wilkinson RJ, Hoal EG, Schurr E. Neutrophils: Innate effectors of TB resistance? *Front Immunol* (2018) **9**:2637. doi:10.3389/fimmu.2018.02637

Data Availability Statement

The datasets generated for this study are available on request. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Tables

Table 1. Correlation analyses results between cytokine levels and mycobacteria killing rates.

	Culture			**Stimuli**		**Parameters**				**Correlation**	
PMN	MDM	CC	BCG	Map	IL-1β	IL-8	killing	n	coefficient	p
✓	✓	✓	✓	✓	✓	✓	✓	24	0.64	<0.0001
✓	✓		✓	✓	✓	✓		16	0.75	0.0011
✓	✓	✓	✓		✓		✓	12	0.75	0.0074
✓	✓	✓		✓	✓		✓	8	0.95	0.0011
✓	✓			✓	✓		✓	12	-0.7	0.011
✓				✓	✓		✓	4	-0.98	0.016
	✓		✓	✓		✓	✓	8	0.87	0.0044
✓	✓	✓	✓	✓		✓	✓	12	-0.8	0.002