Effect of Paratuberculosis Vaccination on the *in Vitro* Release of Neutrophil Extracellular Traps in a Sheep Model

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Abstract

Background

Vaccination of domestic ruminants against paratuberculosis has shown beneficial results in the control of the disease, even though protection is incomplete. This is partly due to the unawareness about the mechanisms taking part during protective immune response. In this sense, despite of the increased number of evidences that point out the participation of neutrophils in the homologous and heterologous protection, its key role is still unclear. Therefore, we evaluate the effect of paratuberculosis vaccination on the release of neutrophil extracellular traps (NETs), through its quantification and visualization after their *in vitro* infection with *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) and non-related pathogens in a sheep model.

Methods

Ovine neutrophils were obtained from non-vaccinated ($n = 5$) and vaccinated sheep ($n = 5$) and infected *in vitro* with *Map*, *Staphylococcus aureus* (*SA*) and *Escherichia coli* (*EC*). After 4 hours, NETs release was quantified and visualized by fluorimetry and immunofluorescence microscopy, respectively.

Results

NETs components were confirmed by co-localization of extracellular DNA with neutrophil elastase and myeloperoxidase visualized by immunofluorescence. Infection of ovine neutrophils with *Map* ($P > 0.05$), *SA* ($P < 0.001$) and *EC* ($P < 0.01$) induced the formation of NETs before vaccination as well as after 15- and 30-days post-vaccination. However, neither vaccination nor time post-vaccination did modify the generation of NETs.

Conclusions

These results show that the release of NETs did not play a fundamental role in the early homologous and heterologous protective immune response after paratuberculosis vaccination, although, other antimicrobial mechanisms not evaluated in this study may participate in the protective immune response.

Background

Paratuberculosis is a chronic debilitating disease of ruminants caused by the intracellular pathogen *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) that causes substantial economic losses worldwide [1–3]. Current hygienic management and test-and-cull methods are insufficient to reduce the prevalence of paratuberculosis due to its long incubation period and the low sensitivity of diagnostic tests [4, 5]. However, despite not preventing infection, vaccination with approved vaccines has resulted in

a reliable and cost-efficient tool to reduce the onset of the clinical disease in field conditions [6, 7]. Even so, the response of vaccinated animals is not homogeneous and some animals could develop severe intestinal lesions without an explanation being found, since the mechanisms by which vaccination ensure protection are unclear [8–10].

This disease affects intestinal tract; and, specifically, intestinal subepithelial macrophages that are the niche where *Map* survive and multiply [11, 12]. Therefore, numerous *in vitro* studies have focused on the interaction between *Map* and macrophages [12–15] and, lately, associated to vaccination [16, 17]. However, little is known about the possible involvement of neutrophils despite it has been demonstrated that these cells play a key role against intracellular infections [18, 19] such as *M. tuberculosis* and *Map* in murine and bovine models respectively [20, 21]. In fact, neutrophils are the first phagocytes that arrive to the inflammatory site to eliminate the pathogen and prompt the activation of the innate and adaptive immune response [22, 23]. Traditionally, their involvement during paratuberculosis pathogenesis has been ruled out due to their short life-span and the unavailability of the mycobacteria sheltered inside macrophages [24, 25]. Nonetheless, *Map* infection has been related with an early migration of neutrophils to the infection site [21] and a malfunction during neutrophil recruitment [26, 27], which could be related to the lower expression CXCL8/IL8, a chemokine associated with neutrophil recruitment and activation, in blood and tissues from infected cows [28] and the lower proportion of neutrophils in ileal tissues from clinically infected animals [29]. In addition, neutrophil extracellular traps (NETs), based on the extracellular release of chromatin fibers [30], have demonstrated to trap and eliminate a wide range of extracellular pathogens (i.e. *Staphylococcus aureus* and *Escherichia coli*), but also intracellular pathogens such as *Toxoplasma gondii*, *Mycobacterium tuberculosis* and *Map*, preventing their systemic spread [31–35].

The limited comprehension about the mechanisms implicated in the host response against *Map* may constrain the understanding of the protective immune response elicited by paratuberculosis vaccination. In this sense, the role of neutrophils in the *Map* vaccination-induced protective response has been scarcely investigated. However, some studies carried out with murine models have suggested the involvement of neutrophils in the reduction of bacterial burden after bacillus Calmette-Guérin (BCG) vaccination, while others have highlighted the negative impact of neutrophils on the development of a long-lasting immunity [36–38]. Nevertheless, it has been shown that infection of neutrophils from vaccinated rabbits against paratuberculosis give rise an improved responsiveness (i.e phagocytosis, NETs release, etc) of these cells against *Map* and other non-micobacterial pathogens during the first three months after vaccine administration [39]. Due to the lack of knowledge about this issue in ruminants, the objective of this study was to investigate the *in vitro* effect of paratuberculosis vaccination on the response of ovine neutrophils incubated with different pathogens, mediated by the release of NETs.

Results

Visualization of NETs

The induction of NETs was checked using immunofluorescence staining through the co-localization of DNA, neutrophil elastase (NE) and myeloperoxidase (MPO) (Fig. 1). Non-stimulated neutrophils showed a compact multilobed segmented nucleus visible after 4',6-diamidino-2-phenylindole (DAPI) staining, along with the presence of MPO and NE inside the intact cytoplasm (Fig. 1). Besides, nucleus of neutrophils stimulated with Zymosan had a similar appearance that those non-stimulated, although some neutrophils showed NETs, recognized as extracellular structures similar to strands of different sizes stained with DAPI and co-localized with NE and MPO (Fig. 1). *Map*-infected neutrophil shapes were also similar to non-stimulated although few NET-like structures were also observed (Fig. 1). In contrast, different grades of nuclear swelling and rupture were noticed in neutrophils after infection with *Staphylococcus aureus* (SA) and *Escherichia coli* (EC). In this sense, neutrophil cultures infected with SA showed a great quantity of disrupted cells releasing DNA and MPO into the extracellular space (Fig. 1). Some neutrophils seemed to induce NETs, identified as fibers composed of DNA and MPO; however, in these cultures NE staining could not be clearly observed due to the non-specific interferences with SA (Fig. 1). Besides, a great number of aggregated neutrophils were visualized in EC-infected cultures, although NET-like structures were clearer than in SA-infected, detected by co-localization of DAPI, MPO and NE (Fig. 1). These observations were similar both in vaccinated and non-vaccinated sheep and in all times tested.

Extracellular DNA quantification

Generalized linear model (GLM) showed that the infection with SA ($P < 0.001$), EC ($P < 0.01$) and Zymosan ($P < 0.001$) impacted significantly in the release of extracellular DNA before vaccination as well as at 15 and 30 days post-vaccination, although the time of sampling or vaccination did not exert any effect ($P > 0.05$) (Fig. 2).

Multiple comparison analysis showed that infection with SA (52.44 ± 13.83 %) produced a greater extracellular DNA release than non-stimulated (17.34 ± 5.60 %) and *Map*-infected neutrophils (22.79 ± 12.39 %) before vaccination ($P < 0.01$) and at 15 dpv ($P < 0.0001$) and 30 dpv ($P < 0.0001$). Furthermore, the percentage of extracellular DNA was significantly higher in SA-infected neutrophils than EC-infected (34.51 ± 11.20 %) at 15 dpv ($P < 0.001$) and 30 dpv ($P < 0.0001$). Besides, a greater percentage of extracellular DNA was detected in neutrophils infected with EC in comparison to those neutrophils non-stimulated or infected with *Map* only at 30 dpv ($P < 0.0001$). In contrast, despite the slight increase observed in *Map*-infected neutrophils, no significant differences were observed compared to non-stimulated at any time point ($P > 0.05$).

Discussion

Neutrophils have demonstrated to play a fundamental role during pathogenic mycobacterial infection [40]. This fact has been proved in murine models in which the recruitment of neutrophils to the site of infection led to the clearance of *M. tuberculosis* and the prevention of its dissemination during early stages of infection [20, 41]. However, there is a limited research on the involvement of neutrophils in the protection against paratuberculosis [21, 27, 36, 39, 42]. Thus, this study investigated the *in vitro* early

effect of paratuberculosis vaccination on ovine neutrophils, showing that they were able to generate NETs in response to *in vitro* infection with *Map*, *SA* and *EC* without any association with vaccine up to 30 days post-vaccination.

The release of NETs is characterized by the immobilization and elimination of pathogens, although their presence could act as a double-edged weapon related to severe collateral tissue damage [30, 43, 44]. Previous studies have demonstrated the generation of NETs in response to *SA* [33, 45, 46], *EC* [31, 47] and *Map* [35]. In this study, infection of ovine neutrophils with *SA* resulted in the highest percentage of extracellular DNA. In this context, *SA* infection has shown to promote a rapid extracellular DNA release within the first ten minutes that increased dramatically after four hours in human neutrophils quantified by the same fluorimetric assays [33, 48]. At this time, it has been suggested that the production of NETs reaches its maximum and lysis of neutrophils begins to be appreciated under immunofluorescence microscopy [33]. These finding supports our results which show the higher presence of disrupted neutrophils in *SA*-infected cultures by immunofluorescence staining after four hours of incubation.

Besides, the action of NETs during paratuberculosis pathogenesis has not been fully elucidated. Bovine neutrophils have been demonstrated to release NETs in response to *in vitro Map* infection [35]. Here, although in a lesser extent than *SA* and *EC*, *Map* infection also promoted the release of NETs in ovine neutrophils. This fact was more evident by visualization, while quantification only detected a slightly increase of extracellular DNA in *Map*-infected neutrophils, although this discrepancy could be due to either differences in the methodology or sensitivity between both techniques. Furthermore, it has been reported that NETs are unable to kill *M. tuberculosis*, but they trap the bacteria preventing their dissemination and stimulating the formation of granulomas [32]. Thus, it could be inferred that role of NETs during paratuberculosis infection might be related with the immobilization and, in the short term, the elimination of *Map* enhancing the susceptibility of trapped bacteria to other antimicrobial mechanisms, since it has been observed that NETs release is reduced in the presence of monocyte-derived macrophages through the clearance of their components by phagocytosis [35].

Unlikely to here observed, a higher microbicidal activity against *Map* have been probed in monocyte-derived macrophages from paratuberculosis vaccinated goats [17]. Therefore, it could be feasible that this increase in the innate immune response against homologous stimulation may affect other cells belonging to the innate immune response, such as neutrophils. However, here in, no differences were observed during the first month after vaccination. This could be partly due to the absence of stimuli originated by other resident tissue cells such as macrophages or lymphocytes that may limit the responsiveness of neutrophils in a pure culture. However, these results dissent from those obtained in rabbits in which an increase of NETs release was detected in a pure neutrophil culture at three months after subcutaneous and oral vaccination with inactivated paratuberculosis vaccines [39]. Leaving aside differences in the immune response between rabbits and ruminants, our study analyzed the effect on NET formation only during the first month ant it is possible that changes may appear later on.

Furthermore, an increase of antimicrobial response has been also observed against *M. bovis*, *Corynebacterium pseudotuberculosis*, *SA* and *EC* in paratuberculosis vaccinated rabbits suggesting a possible training effect on neutrophils [39]. The establishment of trained immunity in neutrophils has been previously explored in humans after BCG vaccination, showing a heterologous enhanced protection against non-related pathogens such as *Candida albicans* meanwhile, NETs release was not affected by vaccination [42]. Thus, it is tempting to hypothesize that the protection associated with vaccination is not specifically linked to NETs but to others antimicrobial mechanisms oriented towards the improvement of phagocytosis by trained immunity or the activation of the adaptive immune response since it is claimed that neutrophils are necessary to promote Th1 and Th17 protective response against tuberculosis infection after BCG vaccination [37].

Conclusions

In vitro infection of neutrophils with *Map*, *SA* and *EC* give rise to the release of NETs. However, no effects were detected during the first month after vaccination. Notwithstanding, the effect of vaccination on neutrophils should not be discarded but it is possible that other antimicrobial and immunomodulatory mechanisms may be involve in the early protection against paratuberculosis. Therefore, further studies based on *in vitro* assays and other complementary techniques aimed at determining phagocytosis, cytokine expression or the production of antimicrobial substances are necessary to clarify the role of neutrophils in the immune response elicited after paratuberculosis vaccination.

Methods

Animals

A total of 10 healthy one-year-old female sheep of the rasa aragonesa breed were selected from the experimental flock of the Instituto de Ganadería de Montaña (CSIC-ULE) in Grulleros, León. All sheep were *Map*-free confirmed by two tests: an indirect enzyme-linked immunosorbent assay (ID Screen® Paratuberculosis indirect, IDVet, Grabels, France) and IFN- γ release test (Bovigam® *Mycobacterium bovis* IFN- γ test for cattle, Thermo Fisher Scientific, AG, Switzerland) [49]. Animals were randomly divided in two groups: vaccinated ($n = 5$) and non-vaccinated ($n = 5$). First group was subcutaneously vaccinated with 1 mL of Silirum® (CZ Vaccines, Porriño, Spain), containing 2.5 mg of heat-killed 316F *Map* strain plus high refined mineral oil adjuvant whereas non-vaccinated group was inoculated with 1 mL of phosphate buffered saline (PBS) by the same route. All animals were sampled the day before vaccination as well as 15 and 30 days post-vaccination (dpv). Heparinized-blood was collected in each sampling to isolate neutrophils and carry out two *in vitro* assays.

Ovine neutrophil isolation and culture

Ovine neutrophils were isolated as previously described [50]. Briefly, 100 mL of peripheral blood was collected from the jugular vein into lithium-heparin Vacunatiner™ tubes (Becton Dickinson and Company,

UK) and neutrophils were isolated from the red blood cells layer (RBC) by gradient density centrifugation on Lymphoprep™ (STEMCELL Technologies®, Cologne, Germany). After several washings, neutrophils were resuspended in RPMI1640 medium without phenol red (Gibco®, Paisley, UK), counted in a Neubauer chamber and adjusted at a final concentration of 10^6 cells mL⁻¹. Cell viability determined by trypan blue exclusion and purity estimated by Diff-Quick staining was always higher than 96 % and 90 % respectively.

Subsequently, a total of 2×10^5 neutrophils per well were seeded in 96-well flat-bottom plate (Thermo Fisher Scientific, Rochester, NY, USA) to perform neutrophil extracellular DNA quantification. In addition, an aliquot from each animal of 2×10^5 neutrophils was prepared separately for genomic DNA extraction in order to calculate the percentage of NETs release. To carry out NETs visualization, 10^6 neutrophils per well were seeded on sterile round glass coverslips of 13 mm diameters (VWR, Darmstadt, Germany) previously treated with 0.01 % poly-L-lysine (EMD Millipore Corp, Darmstadt, Germany) in 24-well culture plates (Thermo Fischer Scientific, Rochester, NY) [51]. For both *in vitro* assays, neutrophils were allowed to set for 1 hour at 37 °C and 5 % CO₂ in a humidified incubator before infection.

Bacteria culture and infection

Map K10 reference strain, *SA* and *EC* field strains were kindly provided by NEIKER (Basque Institute for Agricultural Research and Development, Derio, Spain). All strains were prepared as previously described elsewhere [39]. *Map* strain was grown for 3 weeks on 7H9 broth supplemented with 10 % oelic acid-albumin-dextrose-catalase enrichment (OADC), 0.2 % glycerol, 0.05 % Tween 80 and 2 mg/L of mycobactin J (MJ) at 37 ± 1 °C. Besides, *SA* and *EC* were grown on brain heart infusion broth (BHI) for 24 hours at 37 ± 1 °C. After estimation by optical density (600 nm) and colony count on 7H9OADC-MJ (*Map*) and BHI (*SA* and *EC*) agar using 10-fold serial dilutions, bacterial suspensions were adjusted and aliquoted at a concentration of 10^8 colony forming units (CFU) per mL in glycerol: water (1:1) and frozen at -80 °C until required.

Prior to neutrophil infection, aliquots were thawed in fresh 7H9OADC-MJ (*Map*) and BHI (*SA* and *EC*) mediums and incubated for 3 hours at 37 ± 1 °C. Then, bacterial suspensions were centrifuged at $3000 \times g$ for 10 minutes and washed with PBS. Finally, bacterial pellets were resuspended in 1 mL of RPMI1640 medium without phenol red and passed up and down through a 30-gauge needle to disperse de clumps before infection [17].

For both *in vitro* assays mentioned above, neutrophils were infected by duplicate with *Map*, *SA* and *EC* at a multiplicity of infection (MOI) 10:1 (10 bacteria/ neutrophil). Neutrophils stimulated by duplicate with 1 mg mL⁻¹ of Zymosan (Sigma-Aldrich, St. Louis, MO) were used as positive control [52] whereas neutrophils with RPMI1640 medium without phenol red served as a negative control. After infection, neutrophils were incubated for 4 hours at 37°C and 5% CO₂ in a humidified incubator.

Quantification of extracellular DNA

Neutrophil extracellular DNA was quantified as described elsewhere with few modifications [35]. After incubation period, $0.1 \text{ U } \mu\text{l}^{-1}$ of micrococcal nuclease (New England BioLabs, Ipswich, MA) was added to the neutrophils seeded in 96-well plates and incubated for 15 minutes at 37°C in order to disrupt neutrophil extracellular DNA. Afterwards, 5 mM EDTA was incorporated to stop nuclease activity and plates were centrifuged at $300 \times g$ for 15 minutes at 4°C . Extracellular DNA was quantified using Quant-iT™ PicoGreen® kit (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Supernatants were collected and deposited into a new 96-well flat-bottom plate (100 μL) and an equal volume of PicoGreen® (1:200 in 10 mM Tris buffer with 1 mM EDTA) was added to each well. Fluorescence was determined by spectrofluorometric analysis at an excitation wavelength of 488 nm and an emission of 520 nm using automated microplate reader (Biotek® Synergy HT, Biotek Technologies, USA). Total DNA from non-stimulated aliquot of 2×10^5 neutrophils from each animal were extracted using Maxwell® 16 Cell DNA Purification Kit with the Maxwell 16 Instrument (Promega, WI, USA) following manufacturer's protocol and fluorescence intensity was measured as mentioned above. Later on, the percentage of extracellular DNA was calculated by dividing the fluorescence intensity of each sample by the fluorescence intensity of the total homologous genomic DNA [35].

NETs visualization

Neutrophils were washed twice with warm PBS and fixed with 4 % CellFix (BD, Erembodegem-dorp, Belgium) for 20 minutes at 37°C . Thereupon, cells were washed twice with PBS and kept in the same buffer at 4°C until the next day. Then, neutrophils were permeabilized with 0.1 % Triton X-100 (Panreac, Barcelona, Spain) for 10 minutes and blocked with 5 % bovine serum albumin (BSA) (Roche, Mannheim, Germany) for 1 hour at 37°C . For fluorescence staining, neutrophils were incubated with anti-NE primary antibody (ab68672, Abcam, Cambridge, UK) at a dilution of 1:200 in block buffer (Animal-Free Blocker and Diluent, R.T.U, Vector Laboratories, CA, USA) for 1 hour at 4°C . Neutrophils were washed twice with PBS and secondary antibody goat anti-rabbit IgG Alexa Fluor®594 (ab150088, Abcam, Cambridge, UK) was added at a dilution of 1:1000 in block buffer. Cells were kept in the dark for 1 hour at 4°C and washed twice with PBS. Later on, neutrophils were incubated with anti-MPO primary antibody conjugated with Alexa Fluor®488 (bs-4943R-A488, Bioss Antibodies, Woburn, MA) at a dilution of 1:400 in block buffer for 1 hour at 37°C . Afterwards, cells were gently washed twice with PBS and mounted on glass slides using a mounting solution with DAPI medium (Abcam, Cambrigde, UK). NETs visualization was carried out at 600x magnification on a direct microscope (Eclipse Ni-E, Nikon, NY, USA) using appropriate epifluorescence filters. Images were captured using a CMOS scientific camera (Photometrics® Prime BSI™, AZ, USA).

Statistical analysis

Normal distribution of DNA quantification was assessed using Shapiro-Wilk test. Generalized linear model (GLM) with binomial distribution was used to analyze the effect of vaccination, *in vitro* infection and time post vaccination on the log-transformed percentage of neutrophil extracellular DNA release. Then, comparisons were performed using the Student's *t* test with the Tukey-Kramer adjustment for

multiple comparisons. For all analysis, a *P* value of < 0.05 was considered to be statistically significant. Statistical analysis were performed with the R Software 4.0.3 (R Development Core Team).

Abbreviations

BCG: bacillus Calmette-Guérin; BHI: brain heart infusion broth; BSA: bovine serum albumin; CFU: colony forming units; DAPI: 4',6-diamidino-2-phenylindole; dpv: days post-vaccination; *EC*: *Escherichia coli*; GLM: generalized linear model; *Map*: *Mycobacterium avium* subsp. *paratuberculosis*; MJ: mycobactin J; MOI: multiplicity of infection; MPO: myeloperoxidase; NE: neutrophil elastase; NETs: neutrophil extracellular traps; OADC: oelic acid-albumin-dextrose-catalase enrichment; PBS: phosphate buffered saline; RBC: red blood cell layer; *SA*: *Staphylococcus aureus*.

Declarations

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Author contributions

VP, JB, DGE, NAV designed and conducted the experiment. NAV, DEG and MC were involved in the neutrophil isolation, culture and neutrophil extracellular traps quantification and visualization. The manuscript was written by VP, JB, DGE and NAV. The final submitted version was read and approved by all the authors

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Animals handling and blood sampling were conducted in accordance with European (86/609) and Spanish laws (R.D. 223/1988, R.D. 1021/2005 and R.D. 53/2013). Protocols were approved by the Subcommittee on Animal Experiments and Welfare of the University of León (ULE) (OEBA-ULE-016-2017).

Consent of publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Percentage of extracellular DNA estimated by fluorimetric quantification. Bars and vertical lines represented mean percentage values and standard deviations respectively of non-stimulated (C-), Zymosan-stimulated (Zym) and Mycobacterium avium subsp. paratuberculosis (Map), Staphylococcus aureus (SA) and Escherichia coli (EC)-infected neutrophils before vaccination (T0) and 15 (T15) and 30 days (T30) post-vaccination. Significant differences estimated by generalized linear model were expressed as * P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Figure 2

Visualization of neutrophil extracellular traps (NETs) after infection with *Mycobacterium avium* subsp. *paratuberculosis* (Map), *Staphylococcus aureus* (SA) and *Escherichia coli* (EC). NETs were composed by DNA (DAPI; blue), elastase (NE; orange) and myeloperoxidase (MPO; green). White arrow heads point NETs-like structures. Photomicrographs (60x) are representative of both groups (vaccinated and non-vaccinated) and post-vaccination days since no differences were observed between these variables.