

# Characterization of neutrophil extracellular traps in cats naturally infected with feline leukemia virus

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Feline leukemia virus (FeLV), a common, naturally occurring gammaretrovirus in domestic cats, is associated with degenerative diseases of the haematopoietic system, immunodeficiency and neoplasia. FeLV infection causes an important suppression of neutrophil function, leading to opportunistic infections. Recently, a new microbicidal mechanism named NETosis was described in human, bovine and fish neutrophils, as well as in chicken heterophils. The purpose of the present study was to characterize NETosis in feline neutrophils, as well as to evaluate neutrophil function in FeLV naturally infected symptomatic and asymptomatic cats through the phagocytosis process, release of neutrophil extracellular traps (NETs) and myeloperoxidase (MPO) activity. The results showed that feline neutrophils stimulated with protozoa parasites released structures comprising DNA and histones, which were characterized as NETs by immunofluorescence. Quantification of NETs after neutrophil stimulation showed a significant increase in NET release by neutrophils from FeLV<sup>−</sup> and FeLV<sup>+</sup> asymptomatic cats compared with FeLV<sup>+</sup> symptomatic cats. Moreover, the number of released NETs and MPO activity in unstimulated neutrophils of FeLV<sup>+</sup> symptomatic cats were higher than those in unstimulated neutrophils from FeLV<sup>−</sup> and FeLV<sup>+</sup> asymptomatic cats. This study reports, for the first time, NET release by feline neutrophils, along with the fact that NET induction may be modulated by a viral infection. The results indicate that the NET mechanism appears to be overactivated in FeLV<sup>+</sup> cats and that this feature could be considered a marker of disease progression in FeLV infection.

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## INTRODUCTION

Neutrophils are among the first cells involved in the defence against pathogens such as bacteria, fungi and protozoa. These cells utilize mechanisms such as phagocytosis, degranulation and oxidative burst to efficiently kill and eliminate micro-organisms (Nathan, 2006). Recently, a new microbicidal mechanism named NETosis was described in human, bovine and fish neutrophils, as well as in chicken heterophils (Brinkmann *et al.*, 2004; Lippolis *et al.*, 2006; Palić *et al.*, 2007; Chuammitri *et al.*, 2009). In this mechanism, neutrophils die after releasing 'neutrophil extracellular traps' (NETs), threads composed of DNA, histones and granular proteins that not only ensnare microorganisms but also provide a high local concentra-

tion of antimicrobial molecules (Brinkmann *et al.*, 2004; Lippolis *et al.*, 2006; Urban *et al.*, 2006; Guimarães-Costa *et al.*, 2009). The release of NETs is dependent on reactive oxygen species (ROS) production and can be stimulated by bacteria, fungi, protozoa and some soluble molecules (e.g. interleukin-8, lipopolysaccharide,  $\beta$ -glucan and phorbol esters) (Brinkmann *et al.*, 2004; Lippolis *et al.*, 2006; Urban *et al.*, 2006; Fuchs *et al.*, 2007; Chuammitri *et al.*, 2009; Guimarães-Costa *et al.*, 2009).

Feline leukemia virus (FeLV) is a naturally occurring gammaretrovirus found worldwide in domestic cats and some small, related felids (Hoover & Mullins, 1991; Leutenegger *et al.*, 1999). FeLV is a horizontally transmitted virus that can lead to fatal neoplasia, degenerative diseases of the haematopoietic system and immunodeficiency (Dunham & Graham, 2008). There is interest in this

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virus not only for veterinary purposes but also for the treatment of human diseases, as FeLV can be used as an animal model in tumour and AIDS research (Onions, 1985; Miyazawa, 2002).

Cat infection with FeLV has two possible outcomes: approximately 60 % of cats recover from a mild, transient infection with a high titre of neutralizing antibodies, whilst about 30 % fail to develop an effective humoral immune response and become persistently viraemic, leading to immunosuppression and susceptibility to opportunistic infections (Hardy, 1982; Rojko & Kociba, 1991; Dunham & Graham, 2008). Dysfunction of cellular immunity by FeLV infection, however, is not restricted to T or B cells (Cockerell & Hoover, 1977; Tompkins *et al.*, 1989), as the loss of neutrophil function has also been reported as a consequence of FeLV infection. Moreover, neutrophils from FeLV-infected cats suffer reduced ROS production, phagocytosis and chemotaxis (Lafrado & Olsen, 1986; Lewis *et al.*, 1986; Kiehl *et al.*, 1987; Lafrado *et al.*, 1987).

The aim of this study was to analyse NETs from healthy (FeLV<sup>-</sup>) and FeLV naturally infected (FeLV<sup>+</sup>) symptomatic and asymptomatic cats. We have demonstrated for the first time that cat neutrophils release pathogen-induced NETs and that, most significantly, NET induction is associated with the health status of FeLV-infected cats.

## RESULTS AND DISCUSSION

### Neutrophil phagocytosis

Neutrophils are among the first leukocytes to reach the site of infection and play important roles in immune responses to infection. Previous studies have demonstrated that FeLV infection affects neutrophil function and their response to different stimuli (Kiehl *et al.*, 1987; Dezzutti *et al.*, 1989, 1990; Hoffmann-Jagielska *et al.*, 2005). Thus, neutrophils from FeLV<sup>+</sup> experimentally infected and clinically ill cats show a reduced chemotactic response *in vitro* in comparison with neutrophils from FeLV<sup>-</sup> cats (Kiehl *et al.*, 1987). Using flow cytometry, Hoffmann-Jagielska *et al.* (2005) demonstrated that neutrophils from naturally infected animals, although clinically asymptomatic, presented an impaired phagocytic capacity compared with neutrophils from FeLV<sup>-</sup> animals. In their study, although the mean number of bacteria phagocytosed was similar, differences in the percentage of cells able to phagocytose *Escherichia coli* were observed. In our work, the phagocytic capacity of purified neutrophils from FeLV<sup>-</sup>, FeLV<sup>+</sup> asymptomatic and FeLV<sup>+</sup> symptomatic cats was evaluated *in vitro* using *Leishmania* promastigotes. Our results showed that the neutrophils obtained from these different groups were able to bind and phagocytose promastigote forms of *Leishmania* regardless of their FeLV infection status. No difference was observed in the number of parasites phagocytosed per neutrophil (FeLV<sup>-</sup> =  $1.107 \pm 0.06$ ; FeLV<sup>+</sup> asymptomatic =  $1.104 \pm 0.07$ ; FeLV<sup>+</sup> symptomatic =  $1.146 \pm 0.05$ ), in the

percentage of infected neutrophils (FeLV<sup>-</sup> =  $48 \pm 13.5$  %; FeLV<sup>+</sup> asymptomatic =  $49.5 \pm 11.3$  %, FeLV<sup>+</sup> symptomatic =  $47 \pm 13$  %) or in the infectivity index (FeLV<sup>-</sup> =  $26.07 \pm 2.65$ ; FeLV<sup>+</sup> asymptomatic =  $27.02 \pm 2.69$ ; FeLV<sup>+</sup> symptomatic =  $27.62 \pm 2.86$ ) among the studied groups, indicating that the recognition and uptake mechanisms involved in *Leishmania* ingestion by neutrophils are not affected by FeLV infection. These apparently contradictory results could be explained by methodological differences and by the specific micro-organisms used to evaluate neutrophil phagocytic properties, or by the particles utilized, given that *Leishmania* can be recognized by lipophosphoglycan and glycoprotein 63 (gp63) surface molecules (Van Strijp *et al.*, 1993).

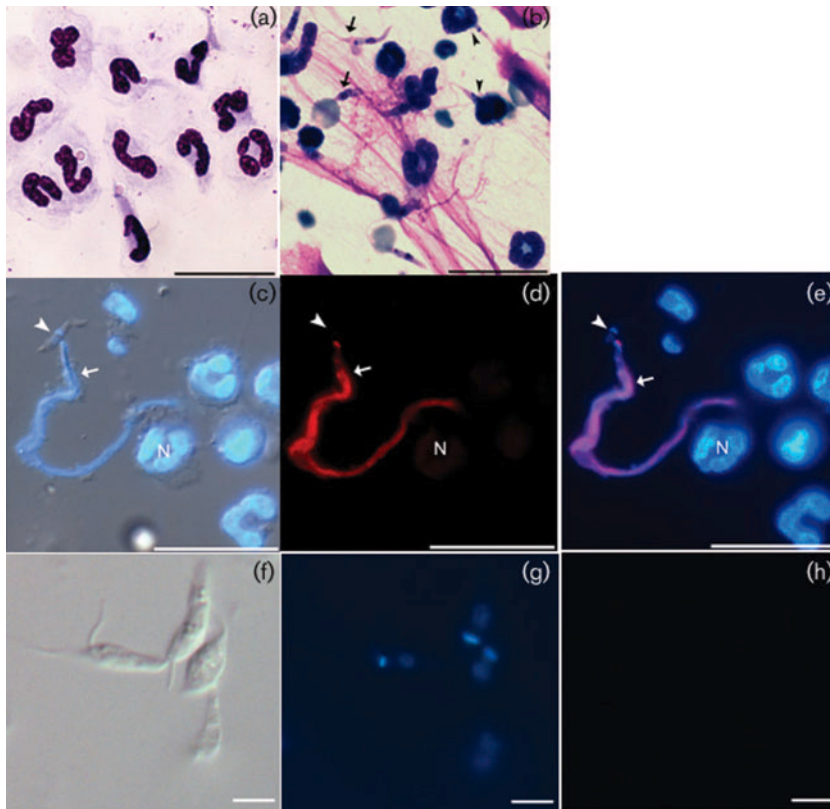
### Characterization of feline NETs

The NETosis process has an important microbicidal function, as demonstrated in human neutrophils during infection with bacteria, fungi and *Leishmania* (Brinkmann *et al.*, 2004; Urban *et al.*, 2006; Guimarães-Costa *et al.*, 2009), as well as in bovine neutrophils during mastitis infection (Lippolis *et al.*, 2006). Generation of oxygen radicals has been associated with NET release (Fuchs *et al.*, 2007). Thus, exogenous H<sub>2</sub>O<sub>2</sub> generated from NADPH oxidase induced NET release in mammals and release of heterophil extracellular traps in chickens (Fuchs *et al.*, 2007; Chuammitri *et al.*, 2009). FeLV infection inhibits neutrophil activation by inhibition of protein kinase C and a decrease in ROS production (Lafrado & Olsen, 1986; Lewis *et al.*, 1986; Lafrado *et al.*, 1987; Dezzutti *et al.*, 1989, 1990; Hoffmann-Jagielska *et al.*, 2005).

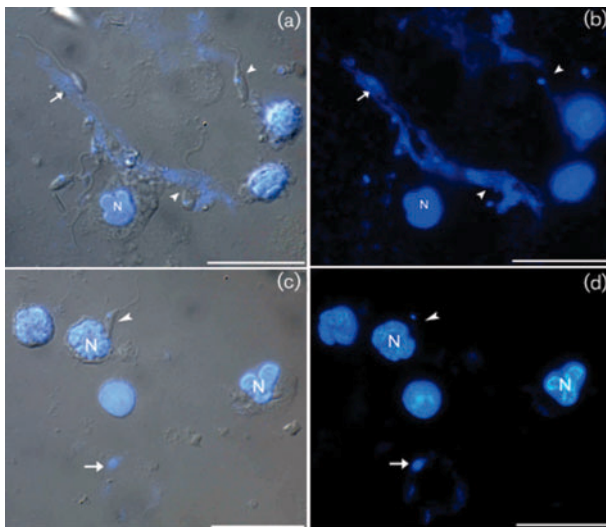
We therefore initially characterized the capacity of healthy cat neutrophils to release NETs following incubation with *Leishmania* promastigotes. A classical NET fibrous structure protruding extracellularly from neutrophils entrapping a number of parasites was observed by optical microscopy, indicating the ability of cat neutrophils to release NETs following *Leishmania* stimulus (Fig. 1a, b). As histone is a known NET-associated component, we stained NETs with 4,6-diamidino-2-phenylindole (DAPI) and anti-H2A histone antibody (Fig. 1c, d). We found promastigotes ensnared by meshes labelled by these two markers (Fig. 1c, d). Secondary control antibody did not label the parasites (Fig. 1h) or the neutrophils (data not shown). To demonstrate the DNA content of these meshes further, DNase was added to the promastigote-cat neutrophil interaction. It was seen that digested NETs disappeared after enzymic treatment, confirming the presence of DNA in NETs released by feline neutrophils (Fig. 2).

### NET release and myeloperoxidase (MPO) activity

Previous studies have shown that FeLV infection impairs neutrophil microbicidal functions and that affected cats are more susceptible to opportunistic infections (Lafrado & Olsen, 1986; Lewis *et al.*, 1986). Decreased activation of



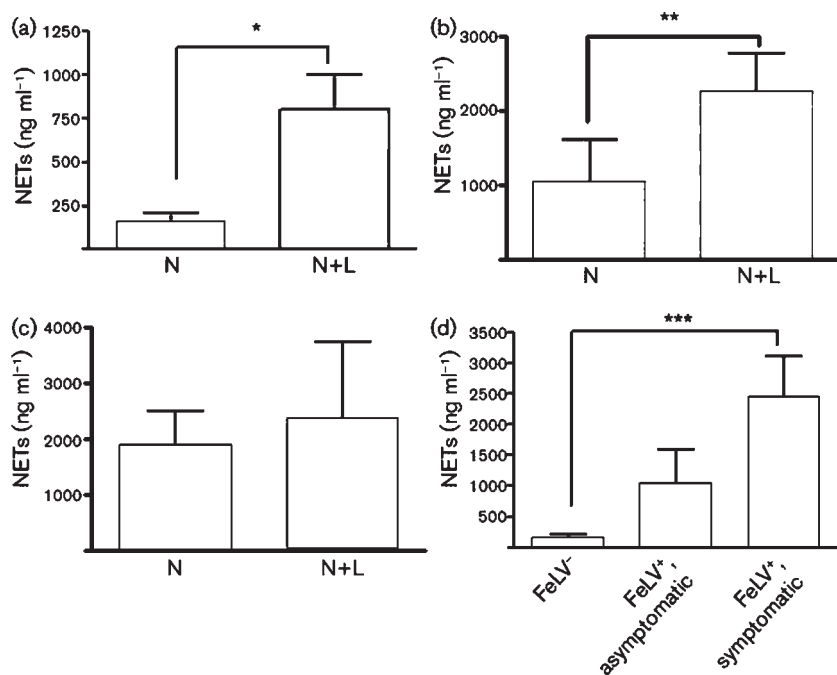
**Fig. 1.** Characterization of feline NETs. (a–e) Cat neutrophils were stimulated with promastigotes (cell:parasite ratio of 1:5) at 34 °C and 5% CO<sub>2</sub> for 2 h. Cells were fixed with paraformaldehyde and stained with Diff-Quick (a, b), DAPI (5 ng ml<sup>-1</sup>) (c) or anti-histone H2A (diluted 1:800) (d). In (c), an overlay of differential interference contrast (DIC) and DAPI fluorescence shows a parasite (arrow-head) ensnared by the NET (arrow). (e) Merged image of DAPI staining (blue) and anti-histone (red) fluorescence showing pink staining resulting from the overlay of images. (f–h) DIC of paraformaldehyde-fixed *Leishmania* promastigotes (f), DAPI staining (5 ng ml<sup>-1</sup>) of the promastigote nucleus and kinetoplast (g) and anti-histone labelling (h). N, Neutrophils. Bars, 20 µm (a–e); 4 µm (f–h).



**Fig. 2.** DNase eliminates feline NET formation. Cat neutrophils were stimulated with promastigotes (cell:parasite ratio of 1:5) at 34 °C and 5% CO<sub>2</sub> for 2 h. Cells were fixed with paraformaldehyde and stained in the absence (a, b) or presence (c, d) of DNase (50 IU ml<sup>-1</sup>) for 2 h and then incubated with DAPI (5 ng ml<sup>-1</sup>). (a, b) DIC (a) and fluorescent image (b) of *Leishmania* entrapped by NETs (arrowheads) stained by DAPI (arrow). (c, d) Phagocytosis of *Leishmania* (arrowhead) and a degraded NET (arrow) following enzymic treatment with DNase shown by DIC (c) and fluorescence (d). N, Neutrophils. Bars, 20 µm.

neutrophils from FeLV experimentally infected animals during phagocytosis of serum-opsonized particles has been described previously (Lewis *et al.*, 1986). Likewise, neutrophils from FeLV<sup>-</sup> animals were not activated by opsonized particles with serum obtained from FeLV<sup>+</sup> cats (Lewis *et al.*, 1986). Dezzutti *et al.* (1989, 1990) demonstrated that neutrophils from experimentally viraemic cats presented reduced protein kinase C activation when stimulated by phorbol myristate acetate (PMA). Similarly, neutrophils from healthy cats exposed to inactivated FeLV or purified p15E FeLV protein resulted in a diminished response to Ca<sup>+</sup> ionophore (Lafrado *et al.*, 1987). Moreover, a reduction in the oxidative activity of neutrophils from FeLV naturally infected asymptomatic animals was observed after stimulation with *E. coli* and/or PMA (Hoffmann-Jagielska *et al.*, 2005).

Thus, we decided to quantify spontaneous (unstimulated) and *Leishmania*-stimulated NET release into supernatants from the neutrophils in the cat groups under analysis. NETs released by neutrophils obtained from FeLV<sup>-</sup> and FeLV<sup>+</sup> asymptomatic cats increased around nine- and threefold after parasite stimulation in relation to unstimulated neutrophils, respectively (Fig. 3a, b). In contrast, neutrophils from FeLV<sup>+</sup> symptomatic cats were not able to significantly increase NET release upon parasite stimulation (Fig. 3c). The same inability to induce NETs was observed in neutrophils from PMA-stimulated symptomatic cats (data not shown). Taken together, these



**Fig. 3.** NET quantification of cat neutrophils. FeLV<sup>-</sup> (a), FeLV<sup>+</sup> asymptomatic (b) and FeLV<sup>+</sup> symptomatic (c) neutrophils were stimulated with *Leishmania* promastigotes for 2 h. Supernatants were recovered and NETs were quantified using a Picogreen dsDNA kit. In (d), basal NETs were measured using unstimulated or resting neutrophil supernatant. Data are expressed as means  $\pm$  SEM. \* $P < 0.002$ , \*\* $P < 0.02$ , \*\*\* $P < 0.001$ ;  $n = 8$  animals per group. N, Neutrophils; N+L, neutrophils plus *Leishmania*.

findings suggest that FeLV infection could lead to an exacerbated neutrophil activation, making these cells unresponsive to new stimuli. Interestingly, when NETs released from unstimulated neutrophils were compared, it was observed that neutrophils from FeLV<sup>+</sup> symptomatic animals produced 15 and 2.3 times more NETs than unstimulated neutrophils from FeLV<sup>-</sup> and FeLV<sup>+</sup> asymptomatic cats, respectively (Fig. 3d).

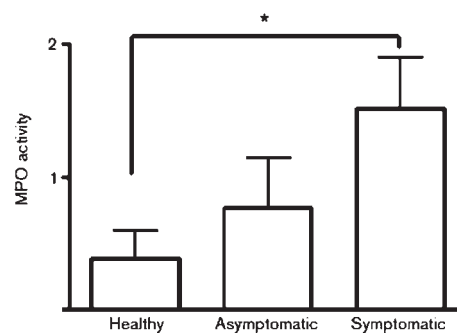
Several granular proteins, such as elastase, MPO, cathepsin G, lactoferrin and gelatinase, are released in association with NETs (Brinkmann *et al.*, 2004). MPO is an important enzyme involved in oxidative stress and in the inflammatory process itself (Loria *et al.*, 2008). Thus, to characterize further the NET release from neutrophils, MPO activity was quantified in the supernatants of unstimulated cells in the different cat groups being studied. The increase in MPO activity was similar to the increase in DNA concentration measured in the unstimulated neutrophils tested (Fig. 4). Furthermore, MPO activity increased in unstimulated neutrophils from FeLV<sup>+</sup> symptomatic animals compared with healthy ones, implying spontaneous neutrophil activation during progressive FeLV infection. Altered neutrophil functions have been detected in other viral infections, particularly with human immunodeficiency virus type 1 and human T-lymphotropic virus type 1 (Guerreiro *et al.*, 2005; Salmen *et al.*, 2007). An increase in NET release and MPO activity in unstimulated neutrophils from FeLV<sup>+</sup> symptomatic cats was also observed, suggesting that progressive viral infections may induce chronic neutrophil activation.

In this report, along with the finding that NET induction may be modulated by a viral infection, NET release by feline neutrophils is described for the first time. Overall,

our results indicate that the NET mechanism appears to be exhausted in FeLV<sup>+</sup> animals, preventing a neutrophil response to other stimuli. We believe that this feature could be considered a marker of disease progression and an important neutrophil trait in FeLV infection, similar to what has been described recently for sepsis and septic arthritis (Margraf *et al.*, 2008; Lögters *et al.*, 2009).

## METHODS

**Animals.** The experimental group consisted of male cats ranging from 1 to 7 years old, which were evaluated with regard to their haemogram, body mass and temperature, dehydration status, appetite, faeces, mucosal cavities, lymph nodes and the presence of tumour mass. None of the cats presented any laboratory or clinical



**Fig. 4.** MPO activity of unstimulated cat neutrophils. FeLV<sup>-</sup>, FeLV<sup>+</sup> asymptomatic and FeLV<sup>+</sup> symptomatic unstimulated neutrophils were used to evaluate the basal MPO activity of supernatants after 2 h incubation at 34 °C and 5% CO<sub>2</sub>. Data are expressed as means  $\pm$  SEM, with  $n = 8$  animals per group. \* $P > 0.05$ .



signs of concurrent infection, including haemotropic mycoplasmas (Bobade & Nash, 1987; Boujon *et al.*, 1991). All animals included in this study were negative for feline immunodeficiency virus (FIV), as detected by immunoblotting for FIV recombinant p24 protein. The animals were separated into three groups ( $n \geq 8$  per group): (i) clinically healthy cats, negative for FeLV; (ii) FeLV naturally infected asymptomatic cats; and (iii) FeLV naturally infected, clinically ill (symptomatic) cats. The FeLV<sup>+</sup> animals were selected using an indirect immunofluorescence kit (VWRD) on blood smears. This work was approved by the Bioethical Committee on Animal Research of the Federal Rural University of Rio de Janeiro (UFRRJ), process #013837.

**Parasite.** *Leishmania amazonensis* (WHOM/BR/75/Josefa) promastigotes were maintained at 26 °C in Schneider's insect medium (Sigma) supplemented with 10 % heat-inactivated fetal calf serum (Ciprion), 10 % human urine and 40 µg gentamicin (Sigma) ml<sup>-1</sup>. In all assays, promastigotes in the stationary phase of growth (5–6 days) were washed twice in PBS (pH 7.2) with centrifugation at 1900 g for 13 min at room temperature, and counted in a haemocytometer.

**Neutrophil purification.** Freshly drawn peripheral blood was collected from each cat by venipuncture from the cephalic vein into tubes containing heparin (Vacutainer; BD). Feline blood was placed on a discontinuous Ficoll-hypaque gradient (densities 1.007/1.119, Sigma-Aldrich) and centrifuged at 400 g for 30 min at 22 °C. Neutrophils were recovered from the 1.119/1.007 interface, resuspended in PBS and washed at 400 g for 10 min. Cells were resuspended in RPMI 1640 without serum and their viability was evaluated by a 0.01 % Trypan blue assay using a haemocytometer.

**Neutrophil–parasite interactions.** Purified neutrophils ( $1 \times 10^5$  per well) were plated onto 0.001 % poly-L-lysine (Sigma-Aldrich)-pre-treated coverslips (13 mm<sup>2</sup>) in 24-well tissue culture plates (Techno Plastic Products). Promastigotes were added to the adhered neutrophils at a parasite:cell ratio of 5:1. After 2 h at 34 °C in 5 % CO<sub>2</sub>, free parasites were removed by extensive washing with PBS and the cultures were stained using Diff-Quick solution and mounted in Permount (Fisher Scientific). The association index was obtained by multiplying the percentage of infected neutrophils by the number of parasites per infected neutrophil, by randomly counting at least 200 cells in each of the duplicate coverslips.

**Characterization of NETs.** Purified neutrophils ( $1 \times 10^5$  per well) were incubated with promastigotes for 2 h, as described above, fixed with 4 % paraformaldehyde and stained with Diff-Quick solution. For immunofluorescence, slides were stained with DAPI (5 µg ml<sup>-1</sup>; Sigma) or anti-histone H2A antibody (diluted 1:150; kindly provided by Dr A. Zychlinsky, Max Planck Institute, Germany), followed by Texas red-labelled anti-mouse (diluted 1:800; Vector Laboratories). Images were taken using an epifluorescence Zeiss Axioplan microscope.

**Quantification of NET released.** Neutrophils ( $5 \times 10^5$  per well) were incubated with or without *L. amazonensis* at a promastigote:neutrophil ratio of 5:1. After 1 h, restriction enzymes (*Eco*R1 and *Hind*III, 20 U ml<sup>-1</sup> each; New England BioLabs) were added to the cultures, which were maintained for 1 h at 34 °C and 5 % CO<sub>2</sub>. After centrifugation, NETs were quantified in the culture supernatant using a PicoGreen dsDNA kit (Invitrogen) according to the manufacturer's instructions. NET DNA concentrations were calculated using herring sperm DNA (Sigma) as a standard.

**MPO activity.** Supernatants (50 µl) from unstimulated neutrophils were incubated with 25 µl 3,3',5,5'-tetramethylbenzidine and 50 µl H<sub>2</sub>O<sub>2</sub>. The colour change reaction was allowed to proceed for 5 min at 37 °C and 100 µl 2 M sulfuric acid was added to stop the reaction.

Different concentrations of lysed neutrophils were used as controls. Absorbance was determined at 405 nm using a microtitre plate spectrophotometer (V-Max; Molecular Devices) with SoftMax PRO 4.0 software.

**Statistical analysis.** Data are presented as means  $\pm$  SEM and were analysed using Student's *t*-test with Kruskal–Wallis or analysis of variance test corrections (GraphPad Prism 3.00).

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## REFERENCES

- Bobade, P. A. & Nash, A. S. (1987). A comparative study of the efficiency of acridine orange and some Romanowsky staining procedures in the demonstration of *Haemobartonella felis* in feline blood. *Vet Parasitol* **26**, 169–172.
- Boujon, C. E., Schärer, V. & Bestetti, G. E. (1991). *Haemobartonella* detection in cat blood smears. *Schweiz Arch Tierheilkd* **133**, 135–136.
- Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y. & Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532–1535.
- Chuammitri, P., Ostojić, J., Andreasen, C. B., Redmond, S. B., Lamon, S. J. & Palić, D. (2009). Chicken heterophil extracellular traps (HETs): novel defense mechanism of chicken heterophils. *Vet Immunol Immunopathol* **129**, 126–131.
- Cockerell, G. L. & Hoover, E. A. (1977). Inhibition of normal lymphocyte mitogenic reactivity by serum from feline leukemia virus-infected cats. *Cancer Res* **37**, 3985–3989.
- Dezzutti, C. S., Wright, K. A., Lewis, M. G., Lafrado, L. J. & Olsen, R. G. (1989). FeLV-induced immunosuppression through alterations in signal transduction: down regulation of protein kinase C. *Vet Immunol Immunopathol* **21**, 55–67.
- Dezzutti, C. S., Lafrado, L. J., Lewis, M. G. & Olsen, R. G. (1990). Inhibition of phorbol ester-induced neutrophil chemiluminescence by FeLV. *Arch Virol* **111**, 75–85.
- Dunham, S. P. & Graham, E. (2008). Retroviral infections of small animals. *Vet Clin North Am Small Anim Pract* **38**, 879–901.
- Fuchs, T. A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., Weinrauch, Y., Brinkmann, V. & Zychlinsky, A. (2007). Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* **176**, 231–241.
- Guerreiro, J. B., Porto, M. A., Santos, S. B., Lacerda, L., Ho, J. L. & Carvalho, E. M. (2005). Spontaneous neutrophil activation in HTLV-1 infected patients. *Braz J Infect Dis* **9**, 510–514.
- Guimarães-Costa, A. B., Nascimento, M. T. C., Fromment, G. S., Soares, R. P. P., Morgado, F., Conceição-Silva, F. & Saraiva, E. M. (2009). *Leishmania amazonensis* promastigotes induce and are killed by neutrophil extracellular traps. *Proc Natl Acad Sci U S A* **106**, 6748–6753.
- Hardy, W. D., Jr (1982). Immunopathology induced by the feline leukemia virus. *Springer Semin Immunopathol* **5**, 75–106.

- Hoffmann-Jagielska, M., Winncka, A., Jagielski, D., Micun, J., Zmudzka, M. & Lechowski, R. (2005). Influence of naturally acquired feline leukemia virus (FeLV) infection on the phagocytic and respiratory burst activity of neutrophils and monocytes of peripheral blood. *Pol J Vet Sci* **8**, 93–97.
- Hoover, E. A. & Mullins, J. I. (1991). Feline leukemia virus infection and disease. *J Am Vet Med Assoc* **199**, 1287–1297.
- Kiehl, A. R., Fettman, M. J., Quackenbush, S. L. & Hoover, E. A. (1987). Effects of feline leukemia virus infection on neutrophil chemotaxis in vitro. *Am J Vet Res* **48**, 76–80.
- Lafrado, L. J. & Olsen, R. G. (1986). Demonstration of depressed polymorphonuclear leukocyte function in nonviremic FeLV-infected cats. *Cancer Invest* **4**, 297–300.
- Lafrado, L. J., Lewis, M., Mathes, L. E. & Olsen, R. G. (1987). Suppression of *in vitro* neutrophil function by feline leukaemia virus (FeLV) and purified FeLV-p15E. *J Gen Virol* **68**, 507–513.
- Leutenegger, C. M., Hofmann-Lehmann, R., Riols, C., Liberek, M., Worel, G., Lups, P., Fehr, D., Hartmann, M., Weilenmann, P. & Lutz, H. (1999). Viral infections in free-living populations of the European wildcat. *J Wildl Dis* **35**, 678–686.
- Lewis, M. G., Duska, G. O., Stiff, M. I., Lafrado, L. J. & Olsen, R. G. (1986). Polymorphonuclear leukocyte dysfunction associated with feline leukaemia virus infection. *J Gen Virol* **67**, 2113–2118.
- Lippolis, J. D., Reinhardt, T. A., Goff, J. P. & Horst, R. L. (2006). Neutrophil extracellular trap formation by bovine neutrophils is not inhibited by milk. *Vet Immunol Immunopathol* **113**, 248–255.
- Lögters, T., Paunel-Görgülü, A., Zilkens, C., Altrichter, J., Scholz, M., Thelen, S., Krauspe, R., Margraf, S., Jeri, T. & other authors (2009). Diagnostic accuracy of neutrophil-derived circulating free DNA (cf-DNA/NETs) for septic arthritis. *J Orthop Res* **27**, 1401–407.
- Loria, V., Dato, I., Graziani, F. & Biasucci, L. M. (2008). Myeloperoxidase: a new biomarker of inflammation in ischemic heart disease and acute coronary syndromes. *Mediators Inflamm* **2008**, 135625.
- Margraf, S., Lögters, T., Reipen, J., Altrichter, J., Scholz, M. & Windolf, J. (2008). Neutrophil-derived circulating free DNA (cf-DNA/NETs): a potential prognostic marker for posttraumatic development of inflammatory second hit and sepsis. *Shock* **30**, 352–358.
- Miyazawa, T. (2002). Infections of feline leukemia virus and feline immunodeficiency virus. *Front Biosci* **7**, d504–d518.
- Nathan, C. (2006). Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* **6**, 173–182.
- Onions, D. (1985). Animal models: lessons from feline and bovine leukaemia virus infections. *Leuk Res* **9**, 709–711.
- Palić, D., Ostojic, J., Andreasenc, C. B. & Roth, J. A. (2007). Fish cast NETs: neutrophil extracellular traps are released from fish neutrophils. *Dev Comp Immunol* **31**, 805–816.
- Rojko, J. L. & Kociba, G. J. (1991). Pathogenesis of infection by the feline leukemia virus. *J Am Vet Med Assoc* **199**, 1305–1308.
- Salmen, S., Montes, H., Soyano, A., Hernández, D. & Berrueta, L. (2007). Mechanisms of neutrophil death in human immunodeficiency virus-infected patients: role of reactive oxygen species, caspases and MAP kinase pathways. *Clin Exp Immunol* **150**, 539–545.
- Tompkins, M. B., Olgivie, G. K., Gast, A. M., Franklin, R., Weigel, R. & Tompkins, W. A. (1989). Interleukin-2 suppression in cats naturally infected with feline leukemia virus. *J Biol Response Mod* **8**, 86–96.
- Urban, C. F., Reichard, U., Brinkmann, V. & Zychlinsky, A. (2006). Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. *Cell Microbiol* **8**, 668–676.
- Van Strijp, J. A., Russell, D. G., Tuomanen, E., Brown, E. J. & Wright, S. D. (1993). Ligand specificity of purified complement receptor type three (CD11b/CD18,  $\alpha$ M $\beta$ 2, Mac-1). Indirect effects of an Arg-Gly-Asp (RGD) sequence. *J Immunol* **151**, 3324–3336.