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Perforin and granzyme A release as novel tool to measure NK cell activation in chickens

Adil Ijaz [a], Femke Broere [a], Victor P.M.G. Rutten [a,b], Christine A. Jansen [c,1], Edwin J.A. Veldhuizen [a,*,1]

[a] Division Infectious Diseases and Immunology, Department Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands
[b] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa
[c] Cell Biology and Immunology Group, Department of Animal Sciences, Wageningen University & Research, Wageningen, the Netherlands

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ABSTRACT

Natural killer (NK) cells are cytotoxic lymphocytes that are present in the circulation but also in many organs including spleen and gut, where they play an important role in the defense against infections. Interaction of NK cells with target cells leads to degranulation, which results in the release of perforin and granzymes in the direct vicinity of the target cell. Chicken NK cells have many characteristics similar to their mammalian counterparts and based on similarities with studies on human NK cells, surface expression of CD107 was always presumed to correlate with granule release. However, proof of this degranulation or in fact the actual presence of perforin (PFN) and granzyme A (GrA) in chicken NK cells and their release upon activation is lacking. Therefore, the purpose of the present study was to determine the presence of perforin and granzyme A in primary chicken NK cells and to measure their release upon degranulation, as an additional tool to study the function of chicken NK cells. Using human specific antibodies against PFN and GrA in fluorescent and confocal microscopy resulted in staining in chicken NK cells. The presence of PFN and GrA was also confirmed by Western blot analyses and its gene expression by PCR. Stimulation of NK cells with the pectin SPE6 followed by flow cytometry resulted in reduced levels of intracellular PFN and GrA, suggesting release of PFN and GrA. Expression of PFN and GrA reversely correlated with increased surface expression of the lysosomal marker CD107. Finally it was shown that the supernatant of activated NK cells, containing the NK cell granule content including PFN and GrA, was able to kill *Escherichia coli*. This study correlates PFN and GrA release to activation of chicken NK cells and establishes an additional tool to study activity of cytotoxic lymphocytes in chickens.

1. Introduction

Natural killer (NK) cells are large granular effector lymphocytes of the innate immune system that play a critical role in surveillance of malignantly transformed and virally infected host cells (Vivier et al., 2008). Additionally, NK cells have also been described to have cytotoxic properties against *T. gondii* (Hauser Jr and Tsai, 1986) and *E.coli* (Garcia-Penarrubia et al., 1989). NK cells act by direct killing of their target cells and by producing cytokines, for example interferon gamma, which causes maturation of dendritic cells (DC) to initiate Th1 immune responses (Moretta et al., 2008). NK cells express a variety of activating and inhibitory receptors (Lanier, 2003) and the dynamic equilibrium of ligand-binding to these receptors determines the response of the NK cell (Vivier et al., 2004). Activation of NK cells leads to degranulation and release of cytotoxic granules containing perforin (PFN) and granzymes in the synapse between target and NK cell. The release of these cytotoxic molecules results in the killing of the target cell (Alter et al., 2004; Topham and Hewitt, 2009).

In chicken, besides being present in blood and spleen, NK cells are a predominant subset of intraepithelial lymphocytes in the gut (Meijerink et al., 2021b). Activation of chicken NK cells is primarily measured by the increased surface expression of CD107 (LAMP-1) (Jansen et al., 2010), or by measuring interferon gamma production (Merlino and Marsh, 2002). Much of the knowledge on chicken NK cells is based on homology with mammalian NK cells. The presence of perforin and granzymes in mammalian NK cells and changes in their intracellular

* Corresponding author.
E-mail address: e.j.a.veldhuizen@uu.nl (E.J.A. Veldhuizen).
[1] Authors contributed equally to this manuscript.

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content after activation has been demonstrated in multiple studies (Jacobs et al., 2001; King et al., 1993; Lee et al., 2018), but this aspect has not been explored in chicken NK cells as yet. For a better understanding of the function of chicken NK cells, it is therefore needed to determine if activation actually leads to release of the active granule compounds PFN and granzymes. The presence of the PFN and granzyme A (GrA) gene has already been described in the chicken genome (Sarson et al., 2008), but determination of their presence in chicken NK cells is lacking.

Chicken PFN consists of 644 amino acids and has an extended C-terminus compared to mammalian PFN (D'Angelo; D'Angelo et al., 2013). In mammals, PFN is predominantly present in NK cells and cytotoxic T cells (Voskoboinik et al., 2010) and is synthesized as an inactive precursor which becomes active when the C-terminus is cleaved off due to the acidic pH in cytotoxic granules (Konjar et al., 2010). The function of PFN is to form pores in the membrane of the target cell, which allows the granzymes to enter the cells and induce apoptosis (Chowdhury and Lieberman, 2008; Gwalani and Orange, 2018). Many studies have demonstrated the crucial role of PFN in NK cell mediated cell death and resistance against development of intracellular viral and bacterial infection in humans (Janka, 1983; Stepp et al., 1999) and mice (Kägi et al., 1996). In contrast to the well described functions of mammalian PFN, chicken PFN has only scarcely been studied, only showing localization within $CD8^+$ T cells of the bursa and spleen using immunohistochemistry (Rauf et al., 2012), and describing gene expression in whole tissues in disease models (Wattrang et al., 2016).

Granzymes, 'granule enzymes', are a group of serine proteases that are involved in apoptosis of transformed cells and virally infected cells (Bovenschen and Kummer, 2010). Granzymes enter into the target cell with the help of PFN and activate various death pathways (Anthony et al., 2010). In humans, granzymes are serine proteases that are expressed in cytotoxic lymphocytes together with PFN (Lieberman, 2003, 2010) and act together functionally. Among different granzymes, GrA is the most important and most widely expressed serine protease in NK and $CD8^+$ T cells (Lieberman, 2010). GrA specifically targets nuclear proteins, for example histones, and causes DNA damage in a caspase independent pathway (Lieberman, 2010; Zhu et al., 2006). In addition to causing programmed cell death, GrA also has a proinflammatory role as it activates pro IL-1β (Van Eck et al., 2017). GrA forms a covalent homodimer which differentiates it from other granzymes (Bell et al., 2003). As for PFN, studies on localization and functionality of chicken GrA are very scarce.

In this study, a commercially available anti-human PFN and anti-human GrA antibody, cross reactive towards the chicken orthologues (Chen et al., 2013) were used to detect the presence of PFN and GrA in chicken cells. We show granular staining of PFN and GrA in NK cells of chickens and that these are released upon activation of the NK cells. This release can be semi-quantified with flow cytometry and inversely correlates with increased surface expression of CD107.

2. Materials and methods

2.1. Identification and alignment of chicken and human PFN, and GrA protein sequences

Amino acid sequences of chicken and human PFN (GenBank IDs: XM_046901804.1 and NM_001083116.3 respectively), and chicken and human GrA (GenBank IDs: AJ544060.1 and NM_006144.4 respectively) were identified using NCBI WGS database https://www.ncbi.nlm.nih.gov/genbank/wgs/. The amino acid sequences of chicken and human PFN and GrA were aligned using ClustalW to determine the identity matrix (Thompson et al., 1994), while signal peptides were predicted with SignalP 4.1 (Petersen et al., 2011).

2.2. Chicken embryonic splenocytes (ED14)

Fourteen-day old embryonated eggs (ED14) from Ross308 chickens were obtained from a commercial hatchery in the Netherlands. Splenocytes at that developmental stage contain a large population of cells that do not express the T Cell Receptor or B cell-specific antigens on their surface and can kill target cells that are susceptible to NK cell mediated killing. This population of embryonic splenocytes thus resemble mammalian NK cells (Göbel et al., 2001; Jansen et al., 2010) and provide an excellent starting cell population for obtaining chicken NK cells. Splenocytes were collected, immediately placed in a petri dish containing ice-cold Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS-/- Lonza, Basel, Switzerland). They were gently squeezed through a 70 μm cell strainer (Corning B.V. Life Sciences, Amsterdam) with a plunger to obtain single cell suspensions, and subsequently centrifuged for 5 min at 208×*g*. The pellet was resuspended in $DPBS^{-/-}$ and lymphocytes were separated by density gradient centrifugation using Ficoll-Paque plus (GE healthcare, the Netherlands) at 355×*g* for 12 min at 20 °C. After harvesting from the gradient, cells were washed two times with cold $DPBS^{-/-}$ and resuspended in pure FCS (Lonza, Basel, Switzerland) at a concentration of 50×10^6 cells per mL. Freezing medium (NK cell medium supplemented with 20% DMSO) was added dropwise and under continuous stirring (final ratio 1:1, v:v). Finally, 1 mL of cell suspension was added in precooled cryovials and stored in a −140 °C freezer for future experiments.

2.3. Cell lines

The chicken T cell line (CU91) (Schat et al., 1992), the chicken B cell line (DT40) (Winding and Berchtold, 2001), and the chicken macrophage cell line (HD11) (Beug et al., 1979) stored at −140 °C were thawed and cultured in appropriate culture media. CU91 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 200 units/mL penicillin, and 200 mg/mL streptomycin (Gibco, Life Technologies Limited, Paisley, UK). DT40 cells were cultured in RPMI-1640 media supplemented with 8% (v/v) FCS, 2% (v/v) chicken serum (Lonza, Basel, Switzerland), 200 units/mL penicillin and 200 mg/mL streptomycin (Gibco, Life Technologies Limited, Paisley, UK). The HD11 cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 200 units/mL penicillin and 200 mg/mL streptomycin (Gibco, Life Technologies Limited, Paisley, UK). All cell lines were cultured at 37 °C and passaged twice a week. CU91 and DT40 cells are suspension cells. The adherent HD11 cells were passaged by detaching cells using 0.25% trypsin/EDTA solution (Gibco, Life Technologies Limited, Paisley, UK). For experiments, all cell lines were used between passage number 2 and 15.

2.4. Anti-PFN and anti-GrA antibodies

The polyclonal antibodies rabbit-anti-recombinant human PFN (14580-1-AP, Proteintech, Sanbio, The Netherlands) and rabbit-anti-recombinant human GrA (11288-1-AP, Proteintech, Sanbio, The Netherlands) were used for detection of PFN and GrA in chicken NK cells. Both antibodies have been successfully used to detect PFN and GrA by Western blot (Li et al., 2021b; Zhang et al., 2018), flow cytometry (Li et al., 2021b), and immunofluorescence (Li et al., 2021a) in human cells. Both anti-PFN and anti-GrA antibodies have shown to have cross reactivity on Western Blot with the chickens orthologs (Chen et al., 2013).

2.5. Detection of PFN and GrA by fluorescence and confocal microscopy

Expression of PFN and GrA in chicken cells was determined by confocal microscopy. For this, chicken NK cells, and cell lines (CU91, DT40) were seeded on poly-L-lysine (Sigma-Aldrich, Merck, St. Luis, MO, USA) coated glass slides in a 24 well plate for 2 h at 4 °C. The adherent cell line HD11 was seeded on glass slides without poly-L-Lysine treatment. After 2 h, supernatants were removed and cells were fixed with 4% paraformaldehyde (PFA) (Alfa Aesar, Haverhill, MA, USA) for 1 h at RT. After fixation, PFA was removed and cells were washed three times

Table 1
Perforin and granzyme A primers.

Gene	GenBank accession	Forward primer	Reverse primer	Fragment length	Annealing temperature
Granzyme A	BU4089623	AGCACCACACTCAAGACCAT	AGATTTGTCCCCATCCTGCT	376	55
Perforin	XM_425355	ATGAAGACCACCTCCCACTG	AATGAGTAGCGGTCCTCCTG	500	60

with DPBS containing 10 mM glycine (Merck Millipore, Burlington, MA, USA) to quench any remaining fixative. Subsequently, cells were permeabilized with 0.5% triton X-100 solution for 15 min followed by blocking with 2% (v/v) chicken serum and 0.05% Tween-20 in PBS for 2 h at 4 °C. Staining for PFN and GrA of NK cells was performed using rabbit-anti-PFN or rabbit-anti-GrA antibodies (1:200) overnight at 4 °C in a humified chamber. The next day, cells were stained with the secondary antibody donkey-anti-rabbit-Alexa488 (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) (1:1000) for 2 h at RT. Nuclear staining was performed with 5 μg/mL DAPI for 5 min at RT. The cells were washed twice with washing buffer containing 0.05% Tween-20 for 5 min after each staining step. Finally, the glass cover slides were mounted on Poly-L-lysine slides (Menzel Glazer GmbH & Co KG, Braunschweig, Germany) using FluorSave (Calbiochem, Merk Millipore, Burlington, MA, USA) mounting medium. Cells were imaged using a Leica TCS SPEII microscope (Leica, Amsterdam, the Netherlands) and images and z-stacks were taken at 100x magnification. Images were analyzed with FIJI software (NIH. Version ImageJ 1.52r).

2.6. Detection of PFN and GrA by Western blot

PFN and GrA protein expression in chicken cells was determined by Western blot. In addition, lysates of Jurkat cells (JC) (human T cells; kind gift of Dr. Bart van den Eshof, Department of Biomolecular Health Sciences, Utrecht University, the Netherlands) was also included on the Western blot. Chicken NK cells stored at −140 °C were thawed and used without additional stimulation. The cell lines CU91, DT40, HD11, and JC were taken from cell culture and used without any stimulation. Briefly, 1×10^6 cells were suspended in ice cold RIPA lysis buffer (ThermoFisher Scientific, Waltham, Massachusetts, USA) containing 1 tablet of cOmplete EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland) per 50 mL buffer for 20 min on ice. Subsequently, cell lysates were centrifuged at 20,000×*g* for 20 min at 4°C. Cell lysates were heated at 90 °C for 10 min in the presence of Laemmli sample buffer (Bio-Rad, Hercules, California, USA) containing β-mercaptoethanol (Sigma-Aldrich, Merck, St. Luis, MO, USA). Thirty μL of sample was loaded on a 10% Tris-glycine gel and proteins were separated at 165 V for 30 min followed by 10 min at 135 V. The proteins were transferred onto a PVDF membrane and then blocked with 2% BSA in DPBS-/- (Lonza, Basel, Switzerland) for 1 h at RT. The membrane was incubated with rabbit polyclonal anti-PFN antibody (1:200 v:v) or anti-GrA antibody (1:1000 v:v) respectively overnight at 4 °C. The next day membranes were stained with swine anti-rabbit-HRP (Dako, Agilent, Santa Clara, USA) as secondary antibody for 1 h. Protein bands were digitally imaged using ChemiDoc MP Imaging system (Bio-Rad, Hercules, California, USA) with ECL super signal solution (ThermoFisher Scientific, Waltham, Massachusetts, USA).

2.7. PFN and GrA gene expression in chicken cells assessed by PCR

RNA was extracted from 2×10^6 chicken NK cells, CU91, DT40, and HD11 cells using the RNeasy isolation kit (Qiagen GmbH, Hilden, Deutschland) using the manufacturer's instructions. Subsequently, cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories B.V., Veenendaal, the Netherlands). PCR reactions were performed using primers specific for PFN and GrA and 500 ng of RNA as input (Table 1). The PCR reaction consists of an initial denaturation at 95 °C for 5 min followed by 40 cycles of 30 s denaturation(95 °C), 30 s annealing (60°C for PFN and 55 °C for GrA) and 1 min elongation (72 °C). After amplification, PCR products were run on 1% agarose gel stained with 5% midori green nucleic acid dye for 1 h. The bands were imaged using the Gel Doc (Bio-Rad, Hercules, California, USA) imaging system.

Table 2
Antibodies panel used for staining of chicken NK cells for surface staining of CD107 and intracellular staining of perforin and granzyme A.

Antibody	Host	Dilution used	Isotype
Perforin	Rabbit	1:200	Polyclonal
Granzyme A	Rabbit	1:500	Polyclonal
CD107-APC	Mouse	1:2000	Monoclonal (IgG1)
CD3-APC	Mouse	1:50	Monoclonal (IgG1)
CD3-FITC	Mouse	1:50	Monoclonal (IgG1)
CD8-PE	Mouse	1:400	Monoclonal (IgG1)
CD8-FITC	Mouse	1:400	Monoclonal (IgG1)
DαR-PE	Donkey	1:1000	
DαR-AF488 *(for IFA)*	Donkey	1:1000	
Zombie aqua fixable		1:400	
Viakrome-808		1:400	

2.8. NK cell stimulation

Frozen chicken NK cells were thawed and resuspended in NK medium at a density of 4×10^6 viable cells/mL. Next, 250 μL of NK cell suspension was stimulated with 250 μL of a pectin with known NK cell activation properties: 'SPE6' (Agrifirm, Apeldoorn,the Netherlands) at increasing concentrations from 0.6 to 2.5 mg/mL in the presence of 1 μL/mL Golgistop (BD Biosciences, the Netherlands) and 0.5 μL mouse-anti-chicken-CD107-APC for the CD107 assay only, and 1 μL/mL Brefeldin A (BD Biosciences, the Netherlands) for intracellular PFN and GrA assay only. Cells were incubated at 37 °C, 5% CO_2 for 4 h. Unstimulated cells were used as negative control and a combination of 100 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 500 ng/mL ionomycin (Sigma Aldrich) (PMA/I) was used as positive control. After incubation, the cells were placed on ice and washed in FACS buffer (DPBS-/-, 0.5% bovine serum albumin; and 0.02% sodium azide) and then stained for flow cytometry as described below.

2.9. Detection of PFN, GrA and CD107 by flow cytometry

Flow cytometry was used to determine surface expression of CD107 and intracellular perforin content in NK cells. For determination of the intracellular PFN and GrA content, stimulated and unstimulated chicken NK cells were transferred to a 96 well plate and stained for surface markers, allowing identification of the NK cells (Table 2) followed by live/dead staining with Zombie Aqua Fixable Dye (Biolegend Inc., San Diego, CA, USA). Subsequently, cells were permeabilized using a solution containing 1 volume of FACS permeabilizing solution, 1 volume of lysing solution (BD Biosciences, the Netherlands) and 8 vol of water for 8 min. After permeabilization, cells were blocked with 2% chicken serum in DPBS-/- (Lonza, Basel, Switzerland) for 30 min at 4 °C. Finally, the cells were stained with primary antibody (anti-PFN or anti-GrA) for 20 min at 4 °C (Table 2). Cells were washed twice with FACS buffer after each staining. After the last washing step, cells were resuspended in 200 μL FACS buffer. Finally, fluorescence of 100,000 total cells was recorded using a cytoflex LX flow cytometer and data was analyzed using FlowJo software v. 10.6 (Flowjo LCC, Ashland, OR, USA). The surface expression of CD107 was determined according to the procedures described

A

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Chicken     MKTTSHCLAFSILLLFHHLPTICPQCHRAVGKSCQV--PPVPGTDIVGSGLDVTTMSLTE   58
HUMAN       --MAARLLLLGILLLLLPLPVP-APCHTAARSECKRSHKFVPGAWLAGEGVDVTSLRRSG   57
               ::: * :.****:  **.    ** *. ..*:     ***: :.*.*:***::  :

Chicken     GQVLAI--TKPTSRHCVVCVDRLHEGRSLLLYNGISKWRAGS-KCRKRSSLATGRKSMKT   115
HUMAN       SFPVDTQRFLRPDGTCTLCENALQEGTLQRLPLALTNWRAQGSGCQRHVTRAKVSSTEAV   117
            .  :         .  *.:* : *:**    *  .:::*** .  *::: : *.  .:  .

Chicken     LVSQTEKMAQNWKVGLRVALHPGMGVALAVAGSHSRVAEFGMQKEQEDRYSFASTELHCV   175
HUMAN       ARDAARSIRNDWKVGLDVTPKPTSNVHVSVAGSHSQAANFAAQKTHQDQYSFSTDTVECR   177
              . :..: ::***** *: :*  .* ::******:.*:*. ** ::*:***::  :.*

Chicken     HYWISASHKPRPSSHFLHAVRSLPPHYTPTTAPDYADLLAAYGTHYIHSAQLGGLLRSIT   235
HUMAN       FYSFHVVHTPPLHPDFKRALGDLPHHFNASTQPAYLRLISNYGTHFIRAVELGGRISALT   237
            .* : . *.*    .* :*: .** *:. :* * *  *:: ****:*::.:*** : ::*

Chicken     AIRSCRAAMMGAGTQEVADCLSAEMTANMKS-AAFSAMSSACRKAKSKNSAHSAFHQLFD   294
HUMAN       ALRTCELALEGLTDNEVEDCLTVEAQVNIGIHGSISAEAKACEEKKKKHKMTASFHQTYR   297
            *:*:*. *: *   :** ***:.*  .*:   .::** :.**.: *.*:.  ::*** :

Chicken     ERMVEVEGGEQH--GDLLYG---PPENYAKWLSSLQRLPGLVASIVRPLHTLLPPQDPRQ   349
HUMAN       ERHSEVVGGHHTSINDLLFGIQAGPEQYSAWVNSLPGSPGLVDYTLEPLHVLLDSQDPRR   357
            **  ** **.:   .***:*    **:*: *:.**   ****   :.***.**  ****:

Chicken     AALKAAVSHYIAWRALRVNCSQQCAG---HSAVGLCQCGCAANNAVSADCCSKHRGMAQV   406
HUMAN       EALRRALSQYLTDRARWRDCSRPCPPGRQKSPRDPCQCVCHGSAVTTQDCCPRQRGLAQL   417
             **: *:*:*:: **   :**: *     :*  . *** * .. ..: *** ::**:**:

Chicken     TVTVLEGSGWRGDFFTPTDAYVLLYFNGQRVRTATVWNNNRPRWGARFDFGWVMLHPHQK   466
HUMAN       EVTFIQAWGLWGDWFTATDAYVKLFFGGQELRTSTVWDNNNPIWSVRLDFGDVLLATGGP   477
             **.::. *  **:** ***** *:*.**.:**:***:**.* *..*:*** *:*

Chicken     MKVEVWDQDNGWNDDRLGVCELPVQATGRQQRNVVCFPGGGHLKLSYSAMCGPSLGGSEC   526
HUMAN       LRLQVWDQDSGRDDDLLGTCDQAPKS---GSHEVRCNLNHGHLKFRYHARCLPHLGGGTC   534
            ::::*****.* :** **.*:   ::    .::* *  . ****: * * * * ***. *

Chicken     SEYIPQPPKGQGGLYRLSQWPPKSGDVGPVAWPQHKEGEEEEQEEEEEEEEEEQDNFWE   586
HUMAN       LDYVPQMLLGEPPGN-----------RSGAVW----------------------------   555
             :*:**   *:                . ..*

Chicken     VLGVSEDNRTMVEDEDHQDPSSDLQNPSFNPQNLSVDLKKDPDPRIPFPRYRAAPPAP   644
HUMAN       ------------------------------------------------------------   555
```

B

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Chicken     ---MGVFFTLSTSAAIVLLILPGDLCVDIIGGHEVAPHSRPFMAML--KGKEFCGGALIK   55
Human       MRNSYRFLASSLSVVVSLLLIPEDVCEKIIGGNEVTPHSRPYMVLLSLDRKTICAGALIA   60
                  *:: * *.: **:* *:* .****:**:*****:*.:*   . * :*.****

Chicken     PSWVLTAAHCNLKG-GRVILGAHSRTKREEEEQVIEIAEEIRYPDYCPERKEHDIMLLKL   114
Human       KDWVLTAAHCNLNKRSQVILGAHSITREEPTKQIMLVKKEFPYPCYDPATREGDLKLLQL   120
             .**********:  .:******* *:.*  :*:: : :*: ** * *  :* *: **:*

Chicken     KKRAKINSAVKVIPLPTSGDDLKQGTICSVAGWGQISKRGNKMSDTLREVNVTVISRRIC   174
Human       MEKAKINKYVTILHLPKKGDDVKPGTMCQVAGWGRTHN-SASWSDTLREVNITIIDRKVC   179
             ::****. *.:: **..***:* **:*.*****:  : . . ********:*:*.*:*:*

Chicken     NDKKHYRKEPRITDNMICAGSKRGDKDSCRGDSGGPLICNNVMKGITSFGK-SPCGDPGG   233
Human       NDRNHYNFNPVIGMNMVCAGSLRGGRDSCNGDSGSPLLCEGVFRGVTSFGLENKCGDPRG   239
            **::**. :* *  **:**** **.:***.****.**:*:.*::*:****  . **** *

Chicken     PGVYTRITDKYLKWIRKTIGGDLLTGF                                   260
Human       PGVYILLSKKHLNWIIMTIKGAV----                                   262
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Fig. 1. Sequence alignment and Identity matrix of chicken and human perforin (A) and granzyme A protein (B). Asterisk (*) shows fully conserved residues, colon (:) shows conservation between groups of strongly similar properties, dot (.) indicates conservation of groups of weakly similar properties, Signal peptides are highlighted in yellow, cysteine residues are shown in red, and C-termini are highlighted in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

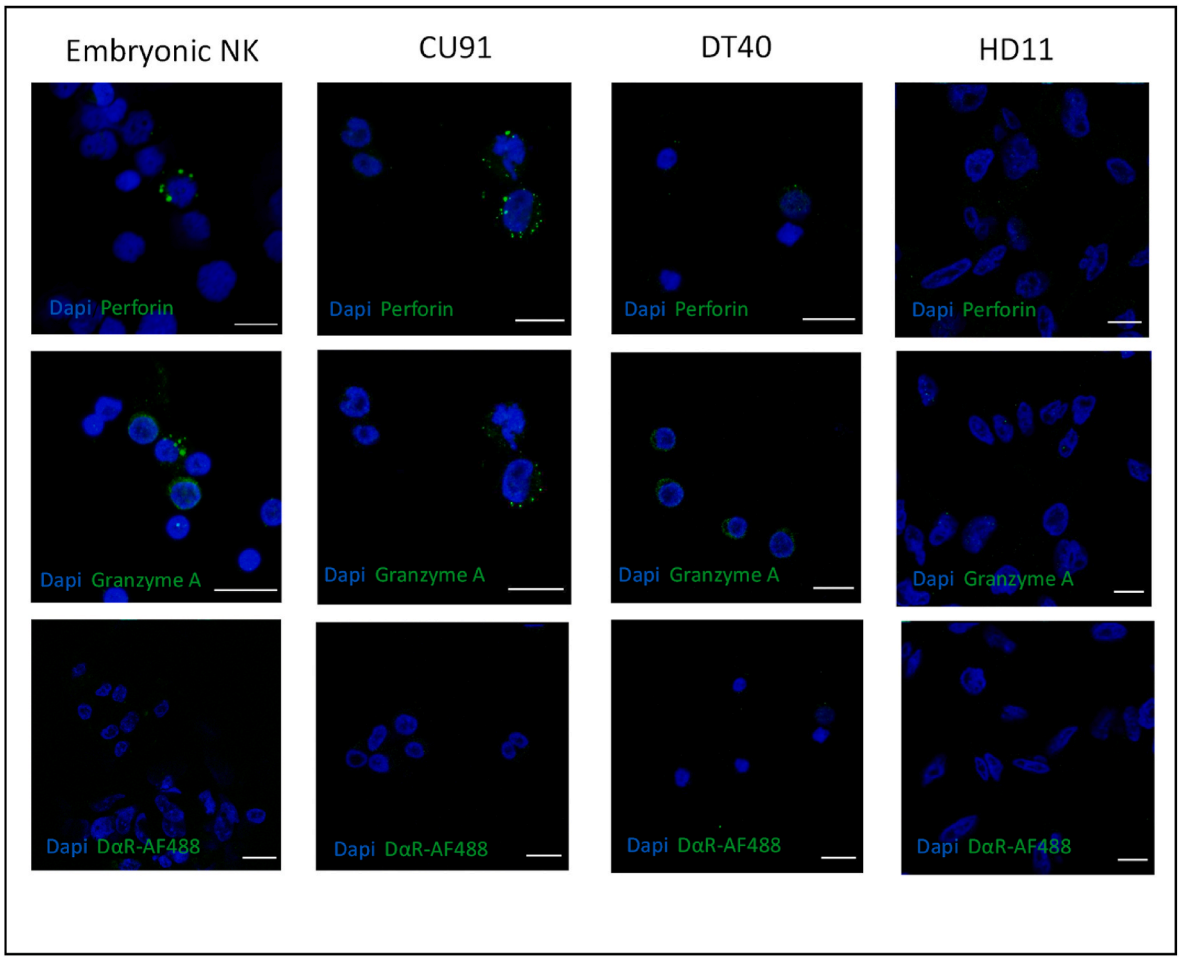

Fig. 2. Perforin and granzyme A are detected in cytoplasm of chicken NK cells. Cells were grown in NK cell culture medium and seeded on glass slides. Cells were blocked, permeabilized and stained with primary antibodies overnight at 4 °C. Perforin and granzyme A are shown in green color while blue is the nuclear staining with DAPI. Scale bar 10 μm, 100x magnification. Bottom row is primary antibody control. Images are representative of 3 independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

previously (Jansen et al., 2010). In short, after 4 h of stimulation, cells were stained with surface markers, and live/dead marker Viakrome808 (Table 2). At least 100,000 total cells were recorded on the cytoflex LX flow cytometer and data was analyzed using FlowJo software v. 10.6 (Flowjo LCC, Ashland, OR, USA).

2.10. Antimicrobial activity of supernatant of activated NK cells

Since PFN and granzymes have been described to have direct antimicrobial activity (Feehan et al., 2022; Ma et al., 2004), the antibacterial activity of supernatant of SPE6 stimulated and unstimulated NK cells was determined. For this, 4×10^6 NK cells/mL were incubated with 0.6–2.5 mg/mL of SPE6 for 4 h. Subsequently supernatant was collected and the 250 μL of supernatant was mixed with 250 μL of 1×10^6 CFU/mL *Escherichia coli* (*E. coli*) in log-phase. After 4 h of incubation, serial ten-fold dilutions were made of the mixture and plated out in triplicate on Trypton Soy Agar plates (Oxoid limited, Hampshire, UK). TSA plates were incubated o/n at 37 °C and the next day viable bacteria were counted.

2.11. Data analysis

All statistical analyses were performed in Graphpad Prism version 9 (San Diego, CA, USA). Statistical significant differences of three independent experiments ($p < 0.05$) between intracellular PFN, GrA and surface expression of CD107 after stimulation of chicken NK cells was determined using one-way ANOVA with Dunnett's multiple comparisons test. Statistical analysis of colony forming units in supernatants of SPE6 treated NK cells and supernatants of unstimulated NK cells was done using one-way ANOVA with Dunnett's multiple comparisons test. The correlation between CD107 surface expression and intracellular PFN and GrA content was assessed using Pearson test.

3. Results

3.1. Comparison of chicken and human PFN and GrA protein sequences

The protein sequences of chicken PFN and GrA share considerable homology with their human counterparts of 38% and 47% respectively. The predicted amino acid sequences of chicken and human PFN and GrA are shown in Fig. 1. Important features of the chicken and human PFN and GrA sequence such as the signal peptide (highlighted in yellow), cystine residues (shown in red), and the C-terminus (highlighted in blue), are indicated in Fig. 1. Chicken PFN has an extended C-terminus as compared to the mammalian counterpart. Signal peptide prediction of chicken and human PFN and GrA is shown in (supplementary) Fig. s1 and s2, respectively. The sequence homology points towards similar functional characteristics of chicken and human PFN and GrA, but also explains the observed cross reactivity of the anti-human PFN and GrA antibodies.

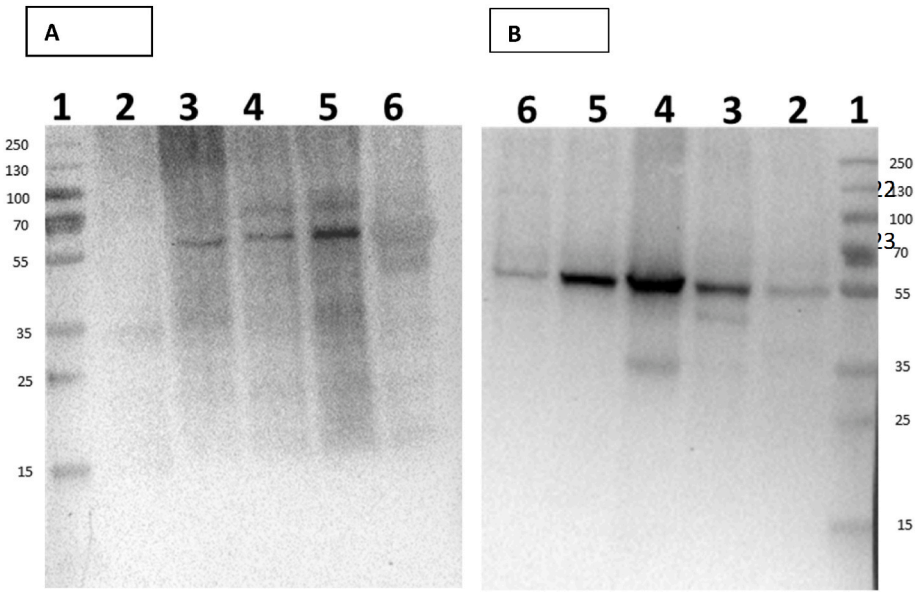

Fig. 3. Perforin and granzyme A protein expression in chicken NK cells and control cell lines detected by Western blot. Lane 1 is marker, lane 2 to 6 are HD11, JC, chicken NK cells, CU91, and DT40 cells respectively. (A) Perforin expression in cell lysates of HD11, JC, chicken NK, CU91, and DT40 cells; (B) Granzyme A expression in cell lysates of HD11, JC, chicken NK, and CU91 cells. Blots are the representative of 3 independent experiments.

3.2. PFN and GrA are present in chicken NK cells

The ability of antibodies to detect PFN and GrA in live cells was determined by immunofluorescence and analyzed by confocal microscopy. A staining in small compartments of the cytoplasm was observed when chicken NK cells were incubated with the anti-PFN and anti-GrA antibodies (Fig. 2). Similar staining was seen in the chicken CU91 cells, while DT40 and HD11 cells did not show any staining. Also no staining was observed in the conjugate control (Fig. 2). Moreover, no signal was observed when non-permeabilized cells were stained with the anti-PFN and anti-GrA antibody (Fig. s3), indicating that the PFN and GrA proteins are located intracellularly. Additionally, using confocal microscopy, the 3D imaging of chicken NK cells and CU91 cells also showed clear staining of PFN protein in specific intracellular compartments, most likely granules (Video s1).

Supplementary data related to this article can be found online at https://doi.org/10.1016/j.dci.2023.105047

3.3. Western blot analysis of PFN and GrA in lysates of chicken NK cells

In addition to the immunofluorescence assay, the presence of PFN and GrA proteins in chicken NK cells was also assessed by Western blot analyses. Lysates of chicken NK cells showed both PFN and GrA expression. On Western blot, the anti-PFN antibody stained a protein band at approximately 70 kDa, corresponding to the calculated mass of PFN, and another, stronger stained band at a lower mass of approximately 60 kDa. Among the cell lines, anti-PFN antibody stained a protein in the lysate of CU91 cells of similar molecular weight based on the size of the product as in chicken NK cells. In lysates of human JC only one protein band appeared at approximately 60 kDa. Anti-PFN antibody did not stain any protein bands in HD11 and DT40 cells (Fig. 3A). Similarly, with the anti-GrA antibody, in lysates of chicken NK cells a protein band at 55 kDa was detected corresponding to a calculated mass of the homodimer of GrA of 58 kDa; and also a second band at approximately 35 kDa. HD11, CU91, DT40, and JC also contained a 55 kDa protein band that was detected by the anti-GrA antibody (Fig. 3B). However, despite numerous efforts to optimize resolution, in our hands both

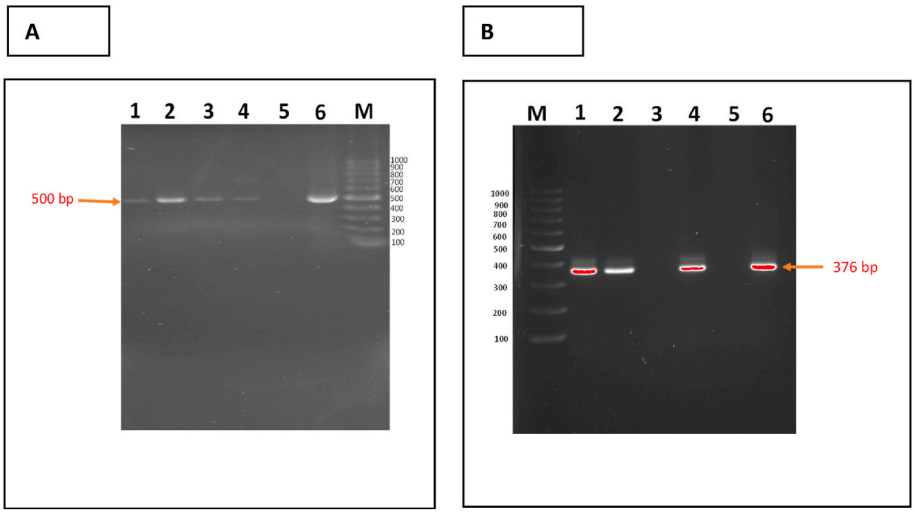

Fig. 4. Gene expression of perforin and granzyme A. qPCR products of chicken NK cells and control cell lines: Lane 1 to 6 are chicken NK cells, CU91 T cells, DT40 cells, HD11 cells, no template control, and positive control (conA stimulated chicken splenocytes) respectively. (A) Gel electrophoresis of qPCR products from perforin reactions, product size 500bp; (B) Gel electrophoresis of qPCR products from granzyme A reactions, 376 bp. Images are representative of 3 independent experiments.

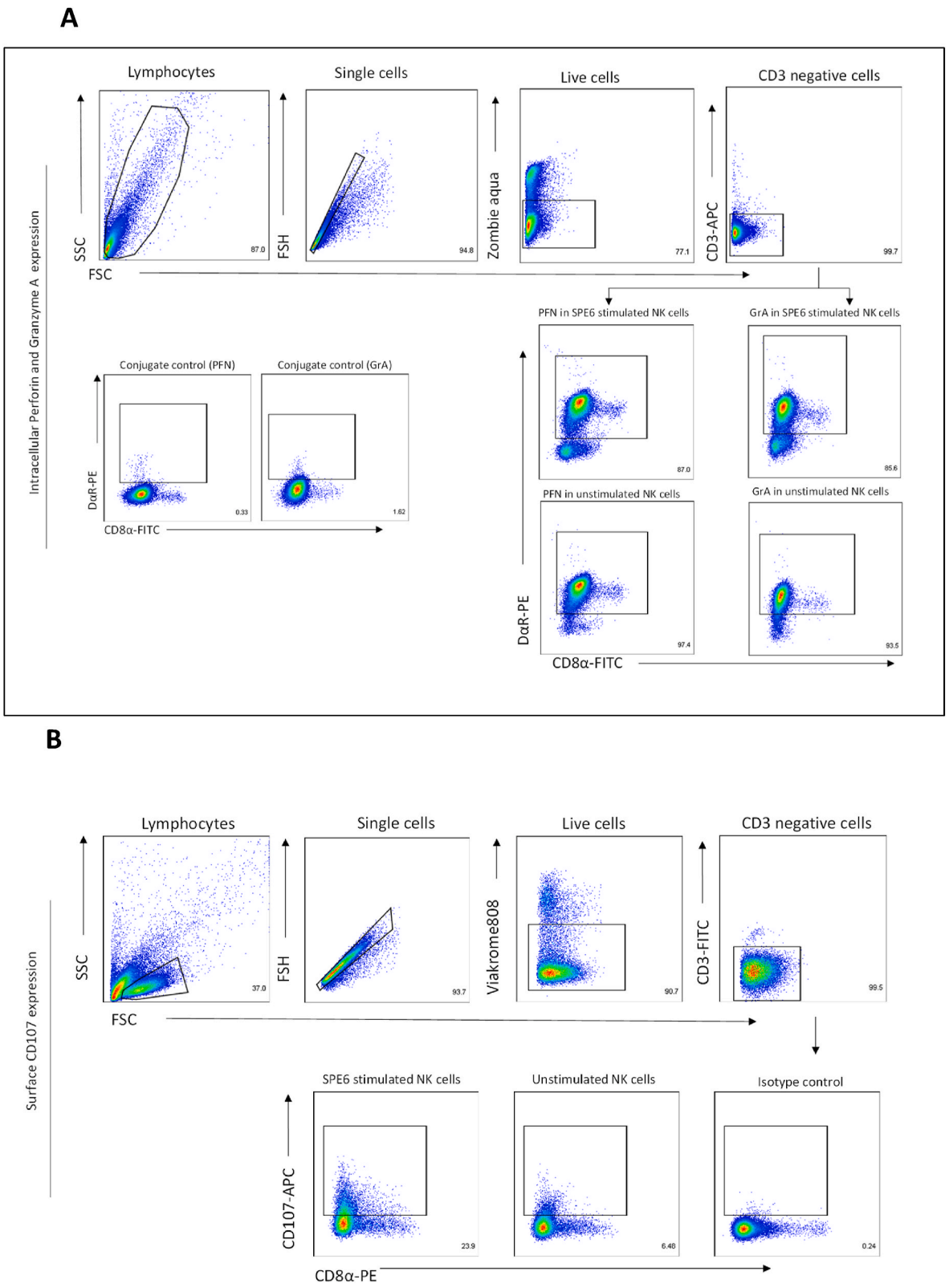

Fig. 5. Representative gating strategy used to analyse intracellular expression of perforin and Granzyme A (A) and surface staining of CD107 in chicken NK cells after stimulation with SPE6 (B) (N = 3 independent experiments).

antibodies showed a relatively high background staining on Western blot, especially for PFN where higher concentrations of antibody had to be used for detection.

3.4. Gene expression of PFN and GrA in chicken NK cells

Gene expression of PFN and GrA was determined by PCR in mRNA isolated from chicken NK, CU91, DT40, and HD11 cells. A cDNA sample of ConA stimulated splenocytes was used as positive control while water

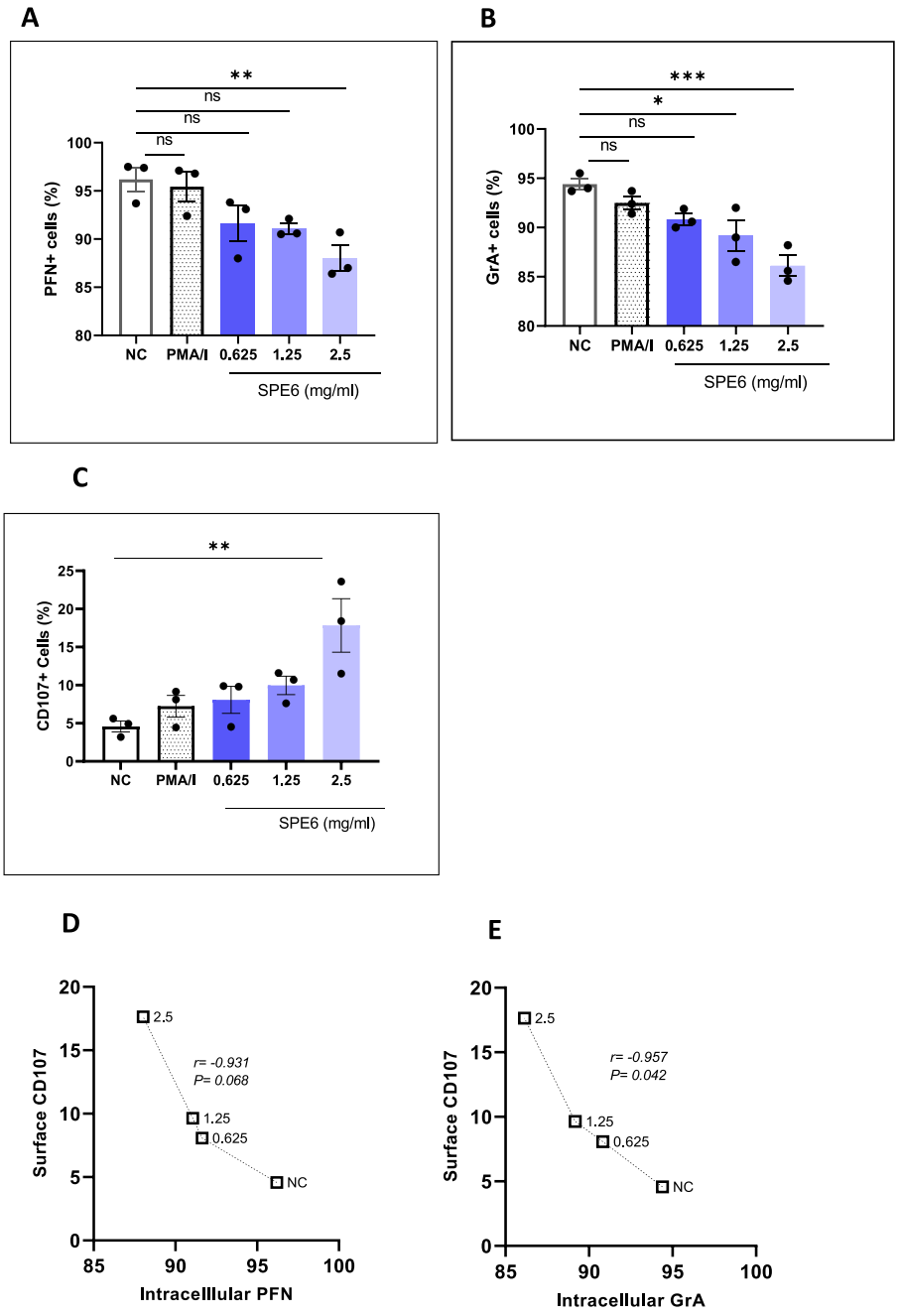

Fig. 6. Intracellular perforin, granzyme A, and surface CD107 expression in chicken NK cells after stimulation with SPE6 (0.6–2.5 mg/mL) for 4 h determined by flow cytometry (N = 3 independent experiments). (5A) Intracellular perforin content in chicken NK cells; (5B) intracellular granzyme A content in chicken NK cells; (5C) surface expression of CD107 in chicken NK cells; (5D) Correlation between surface CD107 expression and intracellular perforin content; (5E) correlation between surface CD107 expression and intracellular granzyme A content.

was used as a no template control. A 500 bp band, corresponding to the calculated size of the specific PFN PCR product was seen for chicken NK cells and the stimulated spleen sample, as well as for all three cell lines (Fig. 4A). The GrA gene analysis showed a band at the calculated size of 376 bp for chicken NK cells, the spleen sample and the CU91, and HD11 cells. DT40 cells did not show GrA gene expression (Fig. 4B). These results show that on a gene expression level both GrA and PFN, as expected, can be detected in NK and T cells, but are not exclusively expressed in these cells.

3.5. Activation of chicken NK cells with SPE6 results in increased surface expression of CD107 and decreased intracellular PFN and GrA content

Flow cytometry was used to determine whether PFN and GrA are released upon activation of NK cells with SPE6. In parallel, surface expression of CD107 was measured. The gating strategy used to identify intracellular PFN an GrA along with surface expression of CD107 in chicken NK cells is shown in Fig. 5. Upon SPE6 stimulation, higher percentages of PFN and GrA negative NK cells were observed compared to the unstimulated control and PMA/I activated NK cells (Fig. 6A&B). Increasing concentrations of SPE6 resulted in decreasing percentages of PFN positive and GrA positive cells. This indicates that PFN and GrA were released upon activation of the NK cells by SPE6, and that this is related to the concentration of the stimulus that was used. Similarly, the surface expression CD107 increased significantly upon treatment with increasing concentrations of SPE6 (Fig. 6C). These results show an inverse correlation between PFN (Fig. 6D) and GrA (Fig. 6E) levels, and CD107 surface expression upon stimulation of NK cells.

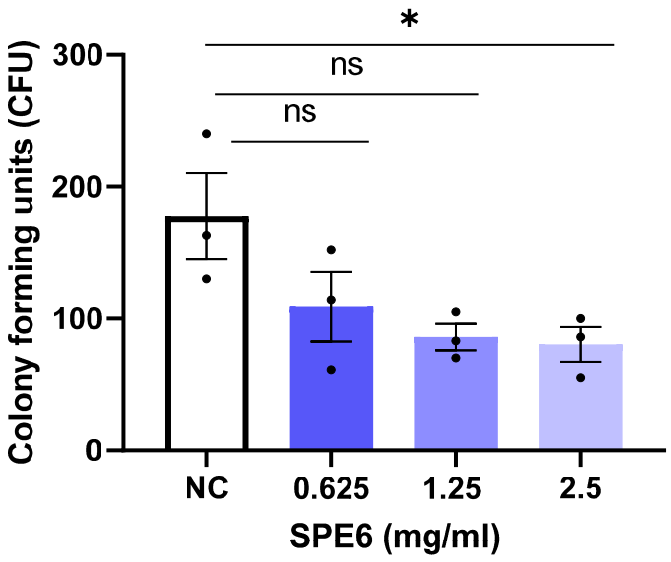

Fig. 7. Decrease in bacterial growth after incubating with supernatants of stimulated chicken NK cells (N = 3 independent experiments).

3.6. Supernatants of SPE6 treated NK cells decrease viability of E.coli

A functional assay to detect the antimicrobial activity of compounds released during NK cell degranulation was performed. Incubation of *E. coli* with supernatants from SPE6 treated NK cells resulted in a decrease in the number of viable bacteria compared to *E. coli* incubated with supernatants of unstimulated NK cells (Fig. 7). The SPE6 itself, still present in the supernatants did not show an antimicrobial effect (Fig. s4). This indicated that antimicrobial compounds, likely including PFN and GrA, are secreted by NK cells upon stimulation with SPE6.

4. Discussion

In this study the presence of PFN and GrA in chicken NK cells and their release upon activation is reported. NK cell biology in chickens, both at the characterization and functional level, is less advanced compared to human and mice due to lack of available tools. The most commonly used functional assay to study NK cell activation in chickens is the lysosomal associated membrane protein-1 (LAMP-1) assay also referred to as CD107 degranulation assay. This assay measures degranulation by detecting the appearance of CD107 on the surface membrane when lysosomes fuse with the cell membrane, as happens during degranulation (Jansen et al., 2010). Based on similarities with studies on human NK cells, surface expression of CD107 was always presumed to correlate with granule release, but this degranulation or in fact the actual presence of PFN and GrA in these granules has never been proven in chicken cells.

Since a previous study already demonstrated cross-reactivity of the polyclonal human anti-PFN and human anti-GrA antibodies with chicken tissues using Western blot (Chen et al., 2013), these antibodies were tested for their ability to visualize PFN and GrA in chicken NK cells and T cells, using confocal and fluorescence microscopy. Both antibodies showed granular staining of PFN and GrA, similar to what has been reported in human and mouse NK (Reefman et al., 2010; Zavan et al., 2015) and T cells (Howden et al., 2019; Park et al., 2020). The presence of PFN and GrA was confirmed by Western Blot and PCR. However, the protein expression of PFN and GrA was not restricted to chicken NK and T cells, since gene expression was also detected in HD11 chicken macrophages (PFN and GrA) and DT40 B cells (PFN) cell lines, while DT40 B cells also showed PFN staining, although to a lower extent. On Western blot PFN and GrA were also detectable but here the specificity for PFN and GrA was not absolute, since background staining of some additional bands was observed. Some are potentially due to breakdown products of PFN and GrA. However, it is not unlikely that specificity on Western blot is lower compared to microscopy because in this technique denatured proteins have to be detected, while antibodies were raised against recombinantly expressed, folded human PFN and GrA. The observation that PFN and GrA are not exclusively present in NK and T-cells is, however, in agreement with human studies where PFN and GrA were shown in macrophages (Van Eck et al., 2017; Xiong et al., 2017). GrA in DT40 cells could also be due to cross reactivity with other granzymes. For example granzyme B is known to be secreted by human B-cells (Hagn and Jahrsdörfer, 2012).

Since PFN and GrA play a critical role in cell mediated cytolysis (Griffiths and Mueller, 1991), and are known to correlate with NK cell degranulation in mammals (Moretta et al., 2008), a functional assay based on intracellular PFN and GrA expression was set up to determine NK cell activation in chickens. Using flow cytometry, cells staining positive for intracellular PFN and GrA, and for cell surface expression of CD107 were quantified. Stimulation of chicken NK cells with the SPE6 increased surface expression of CD107 in a dose dependent manner, presumably correlating with degranulation of NK cells. The ability of oligosaccharides similar to this pectin, to stimulate NK cells (as measured by the CD107 surface expression assay) has been shown before in both human and chicken NK cells (Meijerink et al., 2021a, 2022; Tabarsa et al., 2019). The current study showed that also in chicken cells CD107 surface expression correlates with a lower number of PFN positive and GrA positive cells, strongly indicating that indeed PFN and GrA are released upon activation, similar to mammalian NK cells (Batoni et al., 2005; Krzewski and Coligan, 2012) and cytotoxic CD8 T cells (Sada-Ovalle et al., 2012). The activation of chicken NK cells with a pectin (SPE6) is in agreement with a previous study that showed enhanced activation of intraepithelial NK cells upon stimulation with glucose oligosaccharides (Meijerink et al., 2021a).

Finally, supernatants of stimulated NK cells indeed reduced the number of viable *E.coli* within a few hours, compared to medium control. Although PFN and GrA are best known to target tumorous or virally infected host cells, direct antibacterial activity of PFN and GrA has been described (Feehan et al., 2022; Woodworth et al., 2008). The decrease in the number of bacteria in the present test does not fully prove that this is due to the presence of PFN and granzymes in the supernatants of activated NK cell supernatants. The release of other antibacterial compounds such as antimicrobial peptides upon activation of immune cells could have caused the antibacterial activity. In that aspect it would be an interesting follow up study to determine proper functional activity of chicken PFN and GrA. Although there is a reasonable homology with mammalian orthologs of PFN and GrA, the sequence differences could impact functionality of these proteins with for example higher antibacterial specificity for avian pathogens or avian host cells. Especially the extended sequence of perforin is intriguing and could be related to a functional difference with mammalian PFN. The availability to detect the presence and release of PFN and GrA should aid in these future studies.

In conclusion, this study showed, for the first time, the PFN and GrA protein localization in chicken NK cells, and set up a method to semi-quantify release of these proteins upon NK cell stimulation. Studies on NK cell biology in chickens, or on chicken immunology in general are often hampered by a lack of available antibodies and hence methods as compared to especially human and mice studies. This study added valuable tools alongside the CD107 degranulation assay (Wattrang et al., 2015) and IFN-γ assay (Matsuyama-Kato et al., 2022) to determine more closely the NK and T cell activation. This can be used for studies examining NK and T cell activation on a molecular level both *in-vitro* and *in-vivo* in chickens.

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Author contributions

A.I, E.J.A.V., C.A.J., and F.B., contributed to the design of the experiments; A.I, performed the experiments; A.I, wrote the original draft; A.I., E.J.A.V., C.A.J., F.B, and V.P.M.G.R, reviewed and edited the manuscript; E.J.A.V., C.A.J., F.B, and V.P.M.G.R, supervised the work. All authors have read and agreed to the published version of the manuscript.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2023.105047.

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