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Identification of new populations of chicken natural killer (NK) cells

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ABSTRACT

Natural killer (NK) cell activity is conserved throughout vertebrate development, but characterization of non-mammalian NK-cells has been hampered by the absence of specific mAbs for these cells.

Monoclonal antibodies were generated against *in vitro* IL-2 expanded sorted CD3−CD8α+ peripheral blood lymphocytes, previously described to contain chicken NK-cells. Screening of embryonic and adult splenocytes with hybridoma supernatants resulted in five candidate NK markers.

Activation of chicken NK-cells with PMA/Ionomycin or with the NK target cell-line LSCC-RP9 resulted in increased expression of CD107 (LAMP-1) and a newly developed flow cytometry based cytotoxicity assay showed that NK-cells were able to kill target cells. Combining NK markers with functional assays indicated that marker positive cells showed NK-cell function.

In conclusion, we generated new monoclonal antibodies and developed two functional assays which will enhance our understanding of the role of NK-cells in healthy and diseased chickens.

1. Introduction

Natural killer (NK) cells play an important role in the early defence against intracellular pathogens like viruses, bacteria and intracellular parasites [1,2]. Initially NK-cells were thought to kill any cell that did not express self-major histocompatibility complex (MHC) class I proteins, the so called missing self-hypothesis [3,4]. Now it is widely appreciated that NK-cells express both activating and inhibiting receptors, and that the balance between these signals determines NK-cell activation [2,5]. In addition to their classical role as killers, recently more regulatory functions of NK-cells have been described. Both human and mouse studies suggest that NK-cells may influence the adaptive immune response by the interaction with dendritic cells (DC) or by the production of cytokines [6,7] and a recent study suggests a helper role for NK-cells in eliciting a functional CD8+ T-cell response in the absence of CD4+ T-cell help [8].

In humans, NK-cells have been defined as a population of lymphocytes that lack cell surface expression of CD3 and do express the adhesion molecule CD56 (NCAM) [9,10]. These CD3−CD56+ lymphocytes can be divided into a population of CD56^{bright} cells, which mainly produce cytokines and chemokines and a CD56^{dim} subset which has cytotoxic capacity [11]. Since CD56 is not expressed on murine cells, NK-cells in mice were initially defined by the NKR-P1 family member NK1.1 [12] or by the integrin DX5α [13]. Similar to human NK-cells, also in mice different NK cell subsets have been identified. CD27^{high} NK-cells showed effective cytotoxicity against tumor cell-lines and readily produce IFNγ upon stimulation while CD27^{low} NK-cells are low or non-responsive under the same conditions [14].

In most farm animals, the definition of NK-cells was difficult due to the lack of specific markers [15]. Cow NK-cells for example were defined as CD3−CD2+ lymphocytes [16] and isolation of these cells was based on markers that are not commonly expressed on NK-cells [17].

Recently, Vivier and colleagues have suggested that NKp46, a member of the highly conserved natural cytotoxicity receptor family (NCR) [18], is able to define NK-cells cross-species [19]. Indeed, this receptor has been described to be specific for NK-cells in humans [20], mice [21], monkeys [22], rats [23] and cattle [24] and may be useful in comparative NK-cell analyses between species.

In contrast to mammalian NK-cells, characteristics of non-mammalian NK-cells are lacking. This is mainly due to the lack of NK-cell-specific monoclonal antibodies. Avian NK-cells have been

Abbreviations: CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; CHIRc, hicken Ig-like receptor; ConA, concanavalin A; IBV, infectious bronchitis virus; IEL, intestinal epithelial lymphocytes; LRC, leukocyte receptor region; mAb, monoclonal antibody; MFI, mean fluorescent intensity; NCR, natural cytotoxicity receptors; NK-cell, natural killer cell; PBL, peripheral blood lymphocytes; PMA, phorbol 12-myristate 13-acetate.

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described as a population of cells in the chicken embryonic spleen at a developmental stage where T-cells have not yet migrated to the periphery. These TCR0 cells express surface CD8αα homodimers, but no T- or B-cell-specific antigens and are able to kill the NK-susceptible cell-line LSCC-RP9 [25]. In adult chickens, these avian NK-cells were readily detected in the intestinal epithelial lymphocyte population (IEL) and were used to generate a mAb (28-4). Interestingly, the frequency of avian NK-cells in peripheral tissues was very low, ranging from 0.5% to 1.0% [26]. This is in sharp contrast to NK-cell frequencies in mammals, which have approximately around 10% of NK-cells in blood and spleen.

Based on the differences in NK-cell frequencies between chicken and mammals, one may speculate that chickens simply lack NK-cells in blood and spleen. However, since NK activity in chicken splenocytes has previously been reported [27,28], the absence of chicken NK-cells from other organs than the intestine is not likely. An alternative explanation is that the current markers are not suitable for detection of all chicken NK-cells in blood and spleen.

Interestingly, chicken NK-cells have been reported to express immunoregulatory receptors. These Chicken Ig-like receptors (CHIR) resemble mammalian Ig-like receptors [29] and CHIR genes are located in the chicken genome at a region which was shown to be orthologous to the human leukocyte receptor complex (LRC) [30]. This suggests that chicken NK-cell biology may not be that different from the mammalian NK-cell biology.

Since characterization of chicken NK-cells has been hampered by the lack of specific mAbs, new tools are warranted to study NK-cell biology in the chicken. In this study we set out to identify new markers for chicken NK-cells, which can be used to study the NK-cell frequencies. In parallel, functional assays are essential to confirm that cells which are recognized by NK markers indeed have NK-cell function. Combining markers with functional assays will make it possible to distinguish between presence and functionality of NK-cells in healthy and diseased chickens.

2. Materials and methods

2.1. Animals

One-day-old commercial Lohman Brown chickens were housed in groups and fed ad libitum on commercial feed. Lohman Brown eggs (embryonic day 14) were obtained from a commercial hatchery. Spleens were isolated from 14-day-old embryos and 4-week-old chickens, and homogenised using a 70 μM cell strainer (Beckton Dickinson (BD), Franklin Lakes, NJ, USA) to obtain a single cell suspension. Viable cells were isolated by Ficoll-Paque density gradient centrifugation. Cells were resuspended in IMDM medium supplemented with 2% heat inactivated FCS; 8% heat inactivated chicken serum, 100 U/ml penicillin/streptomycin and 2 mM glutamax I ('NK medium'; Gibco BRL, United Kingdom) and were used directly or cryopreserved until the day of analysis. Embryonic splenocytes were used directly or cultured for up to 7 days in conditioned medium as previously described [25,31]. Conditioned medium was prepared by culturing adult splenocytes (1×10^7/ml) in IMDM supplemented with 0.5% BSA in the presence of 10 μg/ml ConA (Sigma–Aldrich, Zwijndrecht, The Netherlands) for 48 h. The cytokine-containing supernatant was filtered through a 30 kDa filter to remove the ConA (Vivaspin/Sartorius, Weesp, The Netherlands), sterilized by filtration and stored at −80 °C.

For the infectious bronchitis virus (IBV) experiments, 31-day-old SPF layer chickens were either challenged with $10^{4.0}$ EID_{50} of IBV M41 or sterile water by oculo-nasal route, one droplet of 0.05 ml on the eye, one droplet on the nostril. Birds were euthanized using CO_2/O_2 and lungs were collected. Lung tissue was cut into small pieces and digested in RPMI containing 2.4 mg/ml collagenase (Roche Applied Science, Almere, The Netherlands) and 1 mg/ml DNAse (Roche Applied Science) for 30 min at 37 °C, and homogenised using a 70 μM cell strainer. Viable cells were isolated by Ficoll-Paque density gradient centrifugation.

Chickens were housed, handled and treated following approval by the Animal Experimental Committee of the Veterinary Faculty of Utrecht University, The Netherlands. The IBV infection experiment was performed following approval of the Animal Experimental Committee of the GD Animal Health Service, The Netherlands. All experiments were performed in accordance with the Dutch regulation on experimental animals.

2.2. Cell-lines and antibodies

Hybridomas were raised against purified chicken CD3−CD8α+ splenic lymphocytes which were expanded for 2 weeks in the presence of recombinant IL-2 using standard procedures and 48 supernatants were screened. The hybridoma LEP-7 producing the ChCD107 mAb was obtained from the Developmental Studies Hybridoma Bank (DSHB, University of Iowa, IA, United States). The ChCD107 mAb was affinity purified (GammabindPlus, GE Healthcare, Zeist, The Netherlands) from the hybridoma supernatant and biotinylated (D-biotinoyl-ε-aminocaproic acid–N-hydroxysuccinimide ester, Roche Applied Science). Other antibodies used in this study: mouse anti-chicken CD3 (CT3, IgG1), mouse anti-chicken CD8α (CT8, IgG1), mouse anti-chicken CD8β (EP42, IgG2a), mouse anti-chicken CD4 (CT4, IgG1), mouse anti-chicken Bu-1 (AV20, IgG1), mouse anti-chicken γδ-TCR (TCR1, IgG1), mouse anti-chicken αβ1-TCR (TCR2, IgG1), mouse anti-chicken αβ2-TCR (TCR3, IgG1), mouse anti-chicken monocyte/macrophage (KUL01, IgG1), isotype-specific secondary step antibodies goat anti-mouse IgG1, IgG2a and IgG3 (Southern Biotec (SBA), San Diego, CA, USA). Secondary antibodies goat anti-mouse IgG and fluorochrome-labelled streptavidin were obtained from BD.

The chicken B-lymphoblastoid cell-line LSCC-RP9 is commonly used as a chicken target cell-line [32] and was kindly provided by Dr. A. Rebel (CVI, Lelystad, The Netherlands). The chicken B-cell-line 2D8 is not a target for chicken NK-cells. All cell-lines were grown in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin/streptomycin and 2 mM glutamax I.

2.3. Flow cytometry

Flow cytometry was performed to analyse the expression of candidate NK markers on chicken splenocytes. Cells were stained with hybridoma supernatants (mouse IgG) for 30 min at 4 °C, followed by a secondary Ab or isotype-specific antibodies for 20 min 4 °C. Normal mouse serum was used to block a-specific binding followed by staining with T-cell, B-cell and macrophage specific mAbs. CD107 expression was analysed by staining with an anti-ChCD107 mAb, followed by a secondary antibody and when adult splenocytes were used staining with anti-ChCD107 was combined with anti-CD3 and anti-CD8α mAbs. At least 50,000 events were acquired using a FACS Calibur flowcytometer (BD) and data were analysed using the software program CELL Quest (BD) or FlowJO (Threestar Inc., Ashland, OR, USA).

2.4. CD107 assay

The CD107 assay that has been described to study NK-cell activation in humans [33,34] was adapted for the chicken. Embryonic or adult splenocytes were resuspended in NK medium at a concentration of 1×10^6 cells/ml. Cells were stimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml Ionomycin (Sigma) in the presence of 1 μl/ml Golgistop (BD) and anti-ChCD107 mAb during 4 h at 37 °C, 5% CO_2. After incubation, cells were washed in PBS supplemented with 0.5% BSA, stained with a secondary antibody and cell surface markers and flow cytometry was performed. Incubation with anti-ChCD107 or secondary mAbs did not result in a specific staining (data not shown).

2.5. Flow cytometry based cytotoxicity assay

Killing capacity of chicken NK-cells was measured by flow cytometry using the LSCC-RP9 cell-line and the chicken B-cell-line 2D8 as target cells and cultured embryonic splenocytes as effector cells. Target cells were labelled with the fluorescent, cell permeable dye CFSE (5,6-carboxyfluorescein diacetate succinimidyl ester, Molecular Probes, Leiden, The Netherlands) according to the manufacturer's protocol. Briefly, cells were incubated with CFSE for 8 min, after which labelling was stopped using FCS. Cells were washed in PBS and resuspended in RPMI-10 medium supplemented with 10% FCS, penicillin and streptomycin and glutamax. Splenocytes were washed and resuspended in NK medium. Cells were mixed in different effector: target ratios and incubated for 4 h at 37 °C, 5% CO_2. Directly before flow cytometry was performed, propidium iodide (Sigma) was added at a final concentration of 5 ng/ml as well as 10 μl of Flow-Count fluorospheres (Beckman Coulter, Woerden, The Netherlands).

2.6. Statistical analyses

Non-parametric statistical tests were used because the assumption of normally distributed data was not met. Differences between the groups were analysed using Mann–Whitney tests. A *p*-value <0.05 was considered statistically significant. All statistical analyses were performed using the software program SPSS 12.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Screening hybridoma supernatants to identify new candidate markers for chicken NK-cells

In order to identify new markers for chicken NK-cells, splenocytes from 14-day-old embryos were isolated and stained *ex vivo* with supernatants from 47 hybridomas derived against *in vitro* expanded CD3−CD8α+ splenocytes. Based on staining patterns, four groups of markers could be identified as shown in Fig. 1A. Thirty-six supernatants showed minimal reactivity (mean fluorescent intensity (MFI) 2.6, range 1.9–3.2; group 1). Two supernatants resulted a clear population of positive cells (MFI 14.9 (8.6–17.4); group 4) and nine supernatants stain positive, but to a variable extent (MFI 4.3 (3.8–4.7); group 2 and MFI 5.9 (5.3–6.6); group 3).

Next, supernatants were used together with anti-CD3 and anti-CD8α mAbs to stain splenocytes from 14-day-old embryos *ex vivo* and after 7 days of culture. This showed that hybridoma supernatant positive cells were indeed CD3−CD8α+ (Fig. 1B). Furthermore, marker expression changed upon 7 days of culture, as shown in Table 1. Expression of 19 out of 36 markers belonging to group 1 increased, as well as expression of 3 out of 5 (group 2) and 3 out of 4 (group 3). This suggests that different populations of NK-cells may be recognized by these supernatants. These changes in expression were coincided by a decrease in CD8α expression, probably reflecting the activation of chicken splenocytes during culture.

Next, a selection of 12 candidate NK markers representing all groups was further tested on splenocytes from 4-week-old chickens together with the 28-4 mAb. Splenocytes were stained with the hybridoma supernatants in combination with anti-CD3 and anti-CD8α antibodies, and the percentage of marker positive cells in

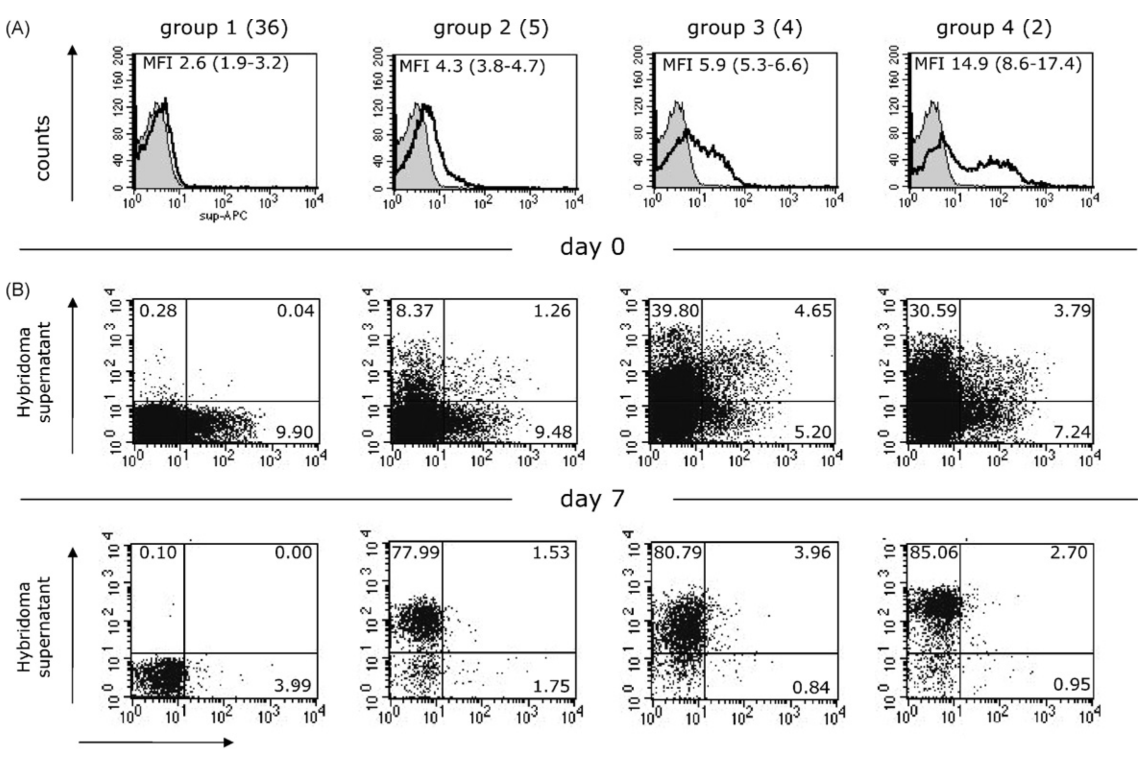

Fig. 1. Initial screening of 47 hybridoma supernatants results in new candidate NK markers. Forty-seven hybridoma supernatants were screened by flow cytometry using splenocytes from 14-day-old chicken embryos. Based on expression, four groups of candidate NK markers could be identified (A). Within the CD3 negative population, expression of NK markers on CD8α positive cells was analysed and representative stainings for each group as defined in (A) are shown. Staining of embryonic splenocytes directly *ex vivo* (A and B) or after culture (C) showed that marker positive cells are CD3−CD8α+ and that the expression of CD8α diminished during culture (C).

Table 1
Expression of candidate chicken NK-cell markers on ED14 cells on day 0 and after 7 days of culture.

Day 0	Day 7								
	Group 1				Group 2			Group 3	Group 4
Group 1	14D9 5D1 10H7 15B11 21D5	7G6 1D11 19H6 1C4 4F8	15E7 7A6 3C6 16B8 1B1	2A4 20C8 14D3 9G4	9G6 13G3 7C1 6H7 1D12	4H3 12H3 6E7 2G3 17B12	6E12 15D1 12A11 7B5	15F8 11F7 4A5	
Group 2	21E3 15C7								5C7 12D7 14H12
Group 3								1G7	14A8 20E5 3C4
Group 4									12D6 6B5

Groups 1, 2, 3 and 4 refer to the grouping that is shown in Fig. 1.

different subsets was analysed. As shown in Fig. 2A and B, the population of CD3−CD8α+ splenocytes (population 1) is small; median 2.0% with a range of 0.7–6.9%. The population of CD3−CD8αdim (population 2) cells is much larger; median 9.2% with a range of 5.8–16.8%. When the expression of the candidate NK markers on CD3−CD8α+ cells was analysed (Fig. 2C), differences between the markers were observed. Staining patterns could be divided into two groups, based on frequencies of positive cells. Eight markers stained the major fraction of CD3−CD8α+ cells (median 35.3, range 19.4–46.6), and five markers were expressed on a minority of the CD3−CD8α+ cells (median 4.8, range 1.5–6.2). The 28-4 mAb stained 2.1% of the CD3−CD8α+ cells (range 0.8–5.5%). Similar results were observed for the CD3−CD8αdim population and CD3− cells (data not shown). Based on these staining patterns which suggested that markers from different groups may recognize different populations of NK-cells, two markers from group 1 and three markers from group 2 were selected for further analyses.

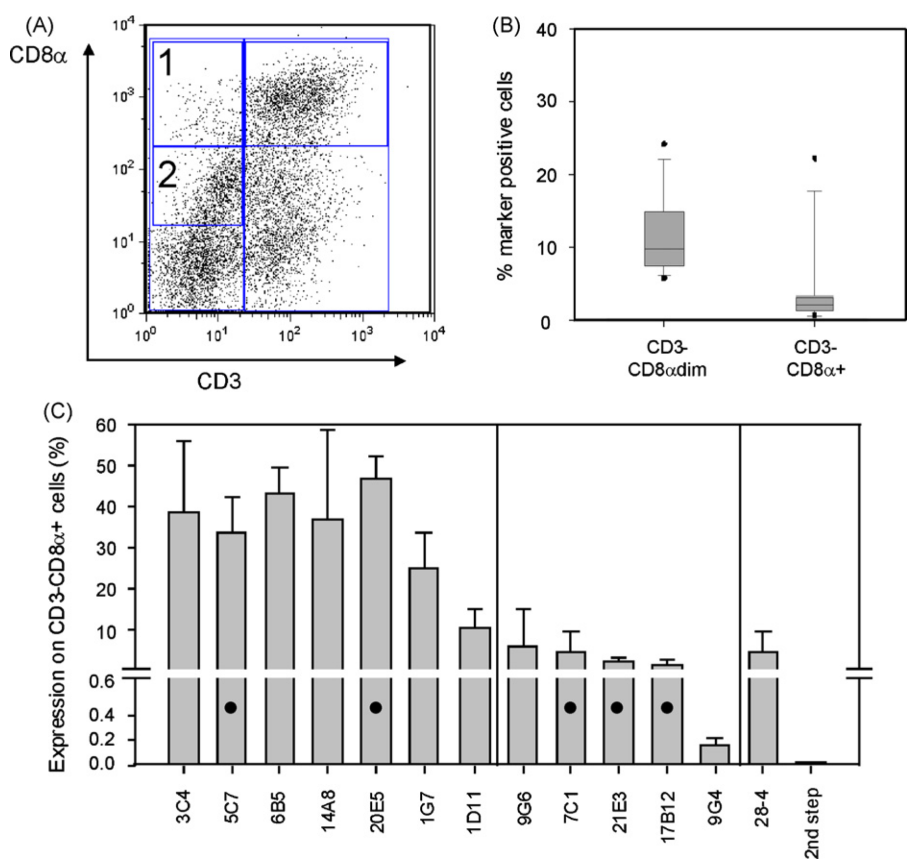

Fig. 2. Additional screening on adult splenocytes results in a panel of five candidate NK markers. Splenocytes from 4-week-old chickens were stained with anti-CD3 and anti-CD8α mAbs. A representative example is shown (A). Frequencies of CD3−CD8α+ cells (population 1) and CD3−CD8αdim splenocytes (population 2) were analysed in 12 4-week-old chickens (B). Co-staining with 12 candidate NK markers (or 28-4, previously described as a marker for intestinal and embryonic NK-cells) and anti-CD3 and anti-CD8α mAbs was performed on splenocytes from 4-week-old chickens. Median expression and interquartile range is shown. Markers that are selected for further study are indicated by a black dot (C).

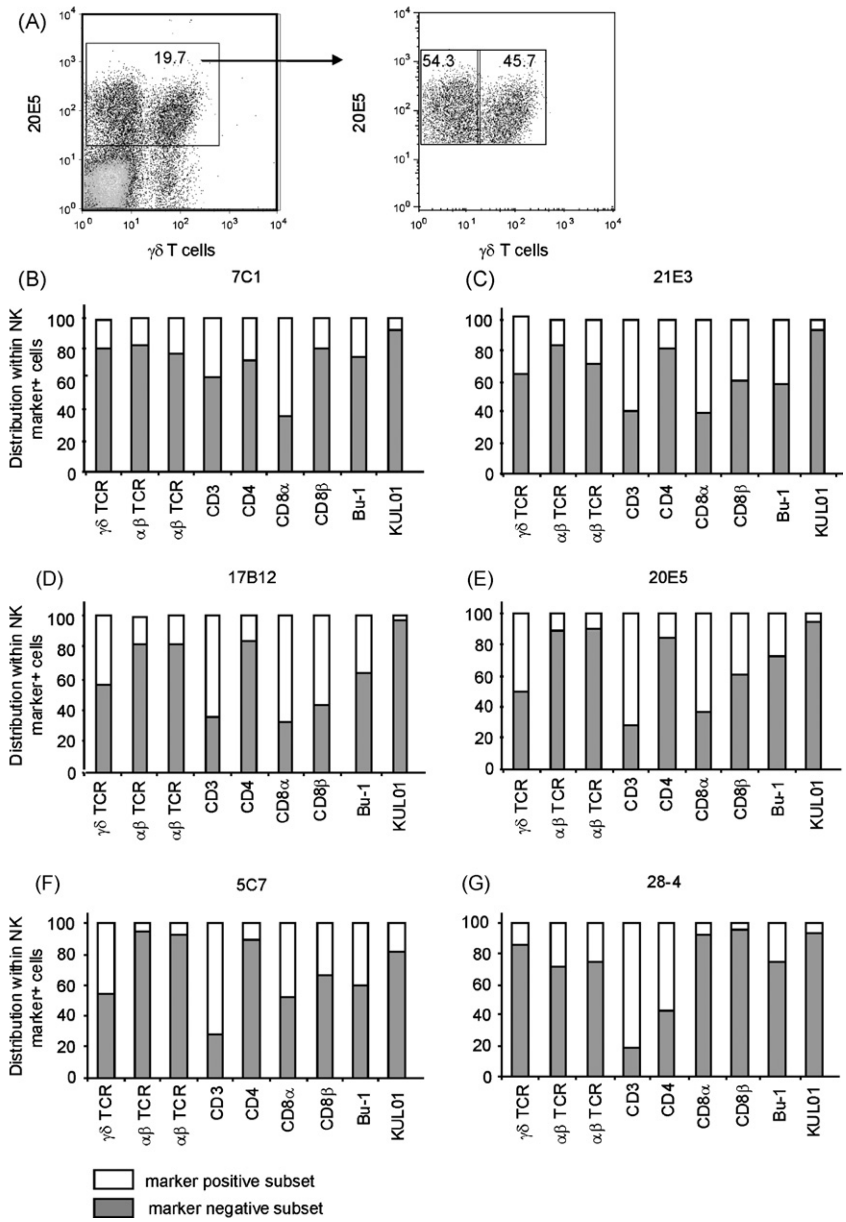

Fig. 3. Co-staining of hybridoma supernatants and non-NK markers. Co-staining with non-NK markers was performed using splenocytes from 4-week-old chickens. NK marker+ cells were selected and within this population frequencies of cellular marker+ and cellular marker− populations were determined by flow cytometry. Co-staining with the candidate NK markers 7C1 (B), 21E3 (C), 17B12 (D), 20E5 (E), 5C7 (F) and the NK mAb 28-4 (G) was performed with a marker for γδ T-cells (TCR1), αβ1 T-cells (TCR2), αβ2 T-cells (TCR3), CD3+ T-cells, CD8α+ cells, CD8β+ cells, CD4+ cells, B-cells and monocytes/macrophages. In grey: median expression in the NK marker negative subset; in white: median expression in the NK marker positive subset.

3.2. Co-staining of hybridoma supernatants and non-NK markers shows that markers are not exclusive for NK-cells

To investigate if the candidate NK markers recognize other cell types as well, co-staining with non-NK markers was performed using splenocytes from 4-week-old chickens. NK marker+ cells were selected and within this population frequencies of cellular marker+ and cellular marker− populations were determined (for a representative example see Fig. 3A showing the percentage of γδ+ and γδ− T-cells within the 20E5+ cells). Co-staining with candidate NK markers and anti-TCR2 and anti-TCR3 mAb shows that all markers are mainly expressed on $\alpha\beta_1$− and $\alpha\beta_2$− T-cells. Co-staining with anti-TCR1 antibodies shows that 7C1 and 28-4 are mainly expressed on γδ− T-cells, while 17B12, 20E5 and 5C7 are expressed in equal amounts on γδ+ and γδ− T-cells. Interestingly, co-staining with anti-CD3 mAbs shows that all candidate NK markers are readily expressed on CD3+ as well as CD3− cells. Co-staining with anti-CD8α mAbs shows differences between the markers: 7C1, 21E3 and 17B12 are predominantly expressed on CD8α+ cells, while the known NK antibody 28-4 is highly expressed on CD8α negative cells. Similar results were observed for staining with anti-CD8β mAb. Co-staining with anti-CD4 mAb shows that the candidate NK markers are predominantly expressed on CD4− cells, except for the known NK marker 28-4. Co-staining with anti-Bu-1 mAb shows that all markers are predominantly expressed on Bu-1− cells. Markers are not expressed on KUL01+ cells. Taken together, differences are observed between the candidate NK markers suggesting that these markers may recognize different populations of NK-cells. Furthermore, co-staining with anti-CD3 mAb will be necessary to distinguish NK-cells from T-cells.

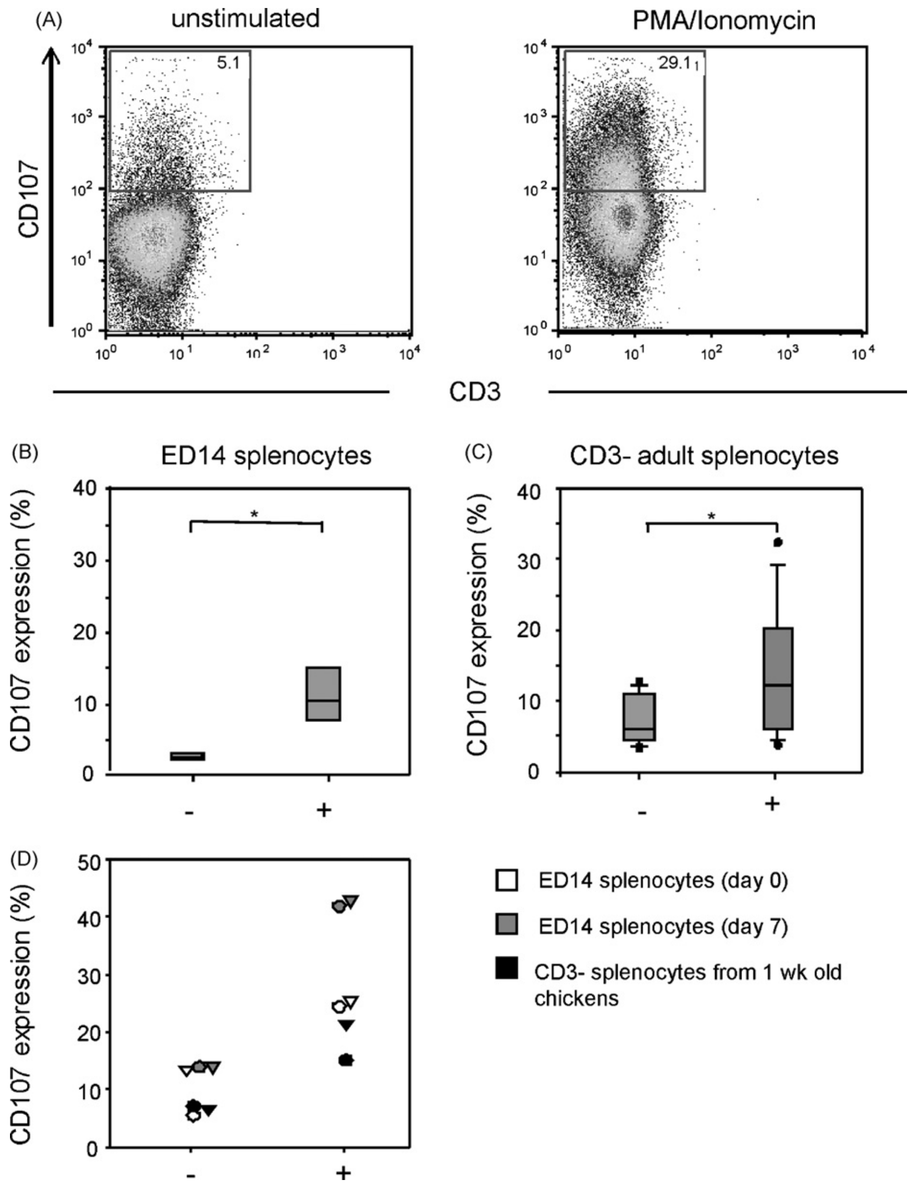

Fig. 4. Expression of ChCD107 (LAMP-1) can be used to measure activation of chicken NK-cells. (A) Embryonic and adult splenocytes were stimulated with PMA/Ionomycin for 4 h and CD107 expression was analysed by flow cytometry. A representative example of CD107 expression in the absence (left panel) and presence (right panel) of stimuli is shown. (B) Stimulation of embryonic splenocytes directly *ex vivo* resulted in a significant increase in CD107 expression ($n = 5$). (C) Stimulation of splenocytes with PMA/Ionomycin results in a significant increase in CD107 expression in CD3− splenocytes ($n = 12$). (D) Co-incubation of splenocytes with the chicken B-cell-line LSCC-RP-9 (NK target cell-line) results in an increase in CD107 expression in NK-cells from different sources ($n = 6$). Significant differences ($p < 0.05$) are indicated by an asterisk.

3.3. Activation of chicken NK-cells is determined by the expression of ChCD107

In order to correlate the newly identified NK markers to functional activities, new assays to measure NK-cell functions were developed. NK-cell activation was measured by analysing the expression of ChCD107 (for a representative example see Fig. 4A). Stimulation of splenocytes from 14-day-old embryos with PMA/Ionomycin in the presence of anti-ChCD107 mAb resulted in an increased CD107 expression (unstimulated median 2.1% (range 1.7–5.1%); stimulated median 8.9% (range 4.6–29.1) $p < 0.05$, Fig. 4B). Similar results were found for splenocytes from 4-week-old chickens, stimulation resulted in a significant increase in CD107 expression in the CD3− cells (unstimulated median 6.0% (range 3.4–12.6); stimulated median 12.3% (range 3.8–32.4), $p < 0.05$, Fig. 4C). Co-incubation of NK-cells with the target cells RP9 also resulted in an increase in CD107 expression in CD3− cells from 14-day-old embryos and 1-week-old chickens (Fig. 4D).

In addition, killing capacity of chicken NK-cells was analysed using a flow cytometry based cytotoxicity assay. Fig. 5A shows killing of the target cell-line RP9 by embryonic splenocytes. This killing is specific for the known chicken target cell-line RP9, because another chicken B-cell-line 2D8 is not killed by these splenocytes (Fig. 5B). Taken together, two assays have been developed that can be used to measure the function of embryonic and chicken NK-cells.

3.4. Combining candidate NK markers with functional assays shows that markers recognize cells with NK function

To investigate if the newly identified NK markers recognize cells which have NK function, staining with NK markers was combined with the measurement of CD107 expression (Fig. 6). In three out of five tested (7C1, 21E3 and 17B12), increased CD107 expression was observed within the marker positive subset upon PMA/Ionomycin stimulation, suggesting that these markers recognize functional chicken NK-cells. Interestingly, two markers with

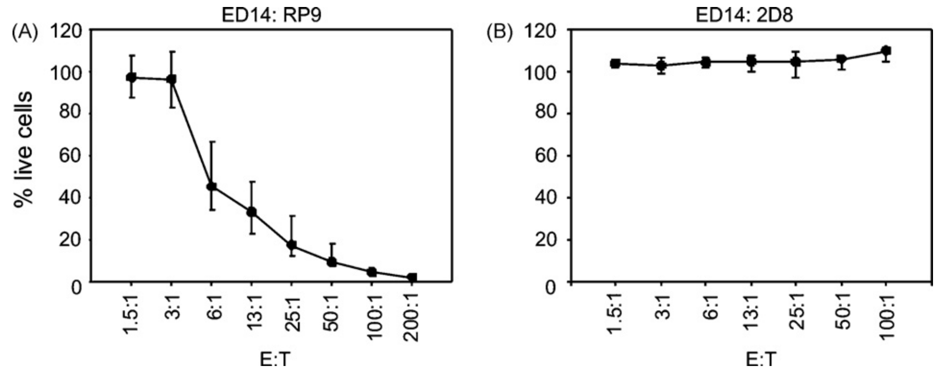

Fig. 5. Flow cytometry based cytotoxicity assay shows killing of target cell-line RP9 by chicken NK-cells. (A) Cytotoxic capacity of cultured ED14 splenocytes was analysed using a flow cytometry based killing assay. The NK target cell-line LSCC-RP-9 was labelled with CFSE and incubated with effector cells at various *E*:*T* ratios. After 4 h, the percentage of live cells was analysed using propidium iodide exclusion. (B) Lack of killing of the chicken B-cell-line 2D8 as target showed that killing of the known NK target B-cell-line LSCC-RP9 was specific. (C) All experiments were performed in triplicate and results from four experiments are shown.

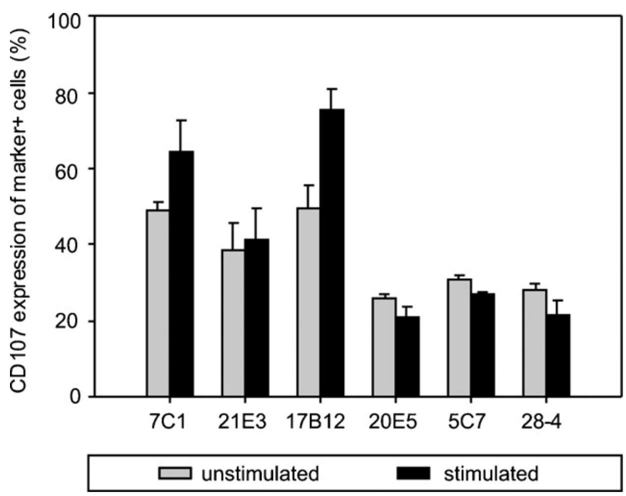

Fig. 6. Co-staining with candidate NK markers and ChCD107 shows that markers recognize cells with NK function. Stimulated splenocytes from 4-week-old chickens were stained with a ChCD107 mAb and a panel of candidate NK markers, as well as 28-4 and expression was analysed by flow cytometry. Three markers (7C1, 21E3 and 17B12) showed increased CD107 expression in marker positive cells upon stimulation. Median expression and interquartile range are shown for four chickens. In grey unstimulated cells, in black stimulated cells.

the highest expression as shown in Fig. 2C (20E5 and 5C7) do not show an increase in CD107 expression in marker positive cells upon stimulation. Furthermore, cells that express the 28-4 mAb that was previously described as a marker for intestinal and embryonic NK-cells do not show enhanced CD107 expression upon stimulation. Taken together, we identified five new candidate NK markers that recognize chicken NK-cells.

3.5. Increased NK-cell activation in lungs of IBV infected chickens

Next, we investigated if the newly developed CD107 assay could be applied to healthy and virus infected birds. To this end, chickens were infected with IBV, lung cells were isolated and CD107 expression was analysed directly *ex vivo* without restimulation as shown in Fig. 7. CD107 expression was significantly higher in CD3−CD8α− cells from IBV infected chickens compared to uninfected chickens (infected median 20.8% (range 14.8–24.6%); uninfected median 7% (range 4.7–11%), $p < 0.05$). Interestingly, no differences in CD107 expression were observed in the CD3−CD8α+ cells (infected median 7.3% (range 5.3–9.7%); uninfected median 6.8% (range 5.5–11.2%)). Thus, *ex vivo* analysis of CD107 expression showed enhanced expression on lung cells from IBV infected chickens, reflecting increased NK-cell activation.

4. Discussion

Characterization of non-mammalian NK-cells has been hampered by the absence of specific mAbs for these cells. Until now, avian NK-cells have been described as a population of cells that express the CD8αα homodimer but lack surface CD3 and Ig [25] and only one NK-cell-specific mAb, 28-4 has been described [26]. Cells with these characteristics have mainly been found in embryonic spleen and the intestinal epithelium of chickens. In contrast, the frequencies of these CD3−CD8α+ avian NK-cells in blood and spleen was very low, ranging from 0.5% to 1% rather than the 10%

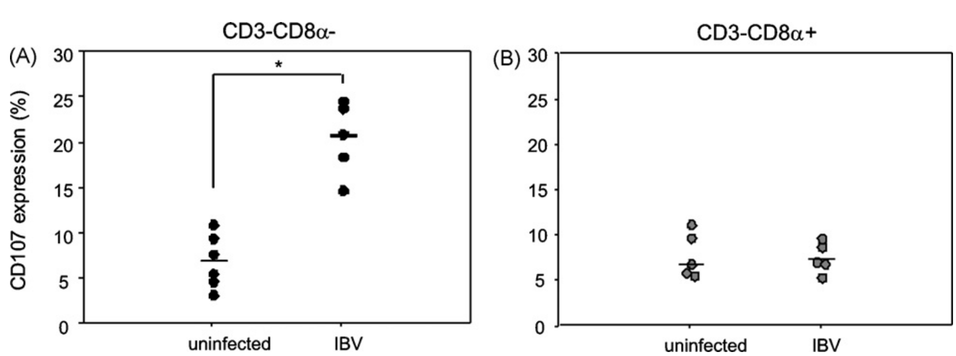

Fig. 7. Increased ChCD107 expression in CD3−CD8α− lung cells after IBV infection. Lung cells from IBV infected and uninfected chickens were isolated and CD107 expression was analysed directly *ex vivo* by flow cytometry. (A) CD107 expression was significantly increased in CD3−CD8α− cells from IBV infected chickens. (B) No differences in CD107 expression in CD3−CD8α+ cells were observed between uninfected and IBV infected chickens. Results for five infected and six uninfected chickens are shown and the vertical bar shows the median CD107 expression. Significant differences ($p < 0.05$) are indicated by an asterisk.

observed in many mammals. This implicates that NK-cells are rarely present outside the gut or more likely that the current markers are not appropriate for the detection of NK-cells in blood and spleen of chickens. Therefore, we set out to identify new markers for chicken NK-cells. Splenocytes from 14-day-old embryos were isolated and stained with supernatants from 47 hybridomas generated against *in vitro* expanded CD3−CD8α+ splenocytes and based on staining patterns four different groups of markers could be identified. Next, staining of the supernatants in the presence of anti-CD3 and anti-CD8α mAbs on splenocytes from 14-day-old embryos confirmed that the positive cells were indeed CD3−CD8α+, which fits earlier observations [34]. Culturing the cells for 7 days with conditioned medium showed changes in expression of the different NK markers and a decrease in CD8α expression. This decrease in CD8α expression may reflect activation of the cells, similar to loss of CD3 expression upon stimulation.

It is tempting to speculate that the differences in expression upon culture are due to the existence of different populations of NK-cells which develop during culture. Staining splenocytes from healthy 4-week-old chickens with a panel of 12 candidate NK markers and the 28-4 mAb resulted in two different groups based on staining patterns within the CD3−CD8α+ population. Interestingly, while in the spleen the population of CD3−CD8α+ cells is rather small (median 2.0%; range (0.7–6.9%)), the population of CD3−CD8αdim cells is much bigger (median 9.2%; range (5.8–16.8%)). As activation of embryonic splenocytes resulted in a decrease in CD8α expression, these CD3−CD8αdim cells may very well represent a population of (activated) NK-cells. This is supported by the similar staining patterns of the candidate NK markers for the CD3−CD8α+ and CD3−CD8αdim population. Furthermore, the total frequency of CD3−CD8αdim and CD3−CD8α+ cells is similar to the frequencies of NK-cells observed in mammals and CD107 is predominantly expressed in CD8αdim cells (data not shown).

Co-staining with non-NK markers suggests that the candidate NK markers recognize different populations. Most markers also recognize CD8α negative cells. As we observed down regulation of CD8α expression upon activation, this suggests that some of these markers may recognize activated NK-cells. Furthermore, staining with the NK markers needs to be combined with staining with anti-CD3 mAbs, similar to the human situation in which the NK marker CD56 is also expressed on T-cells [35]. Some markers may also recognize γδ T-cells (21E3, 17B12, 20E5 and 5C7). In mammals, almost all NK-cell receptors have been found to be expressed by αβ or γδ T-cells [36]. Also bovine γδ T-cells that have been stimulated with IL-15 express the NK-cell receptor NKp46 [37]. Chickens have up to 50% γδ T-cells in blood and spleen [38], and a mAb against a NK receptor from the C-type lectin family (B-NK) recognizes embryonic ED14 splenocytes as well as subsets of splenic αβ and γδ T-cells [39]. This implies that markers for NK-cells may recognize γδ T-cells as well as NK-cells show which is supported by our data showing that the candidate NK markers are also readily expressed on TCR1+ cells.

The function of chicken NK-cells was determined by analysing the expression of CD107 (LAMP-1) which is expressed on the surface of NK-cells upon activation. The expression of CD107 has previously been shown to correlate cytotoxicity and IFNγ production [34] and is commonly used as an assay to measure activation of mammalian NK-cells [34,40]. CD107 expression was found upon stimulation with PMA/Ionomycin and upon stimulation with the target cell-line LSCC-RP9, showing that activation of chicken NK-cells can be measured *in vitro*.

In addition, a flow cytometry based cytotoxicity assay showed killing of the target cell-line LSCC-RP9 by cultured ED14 embryonic splenocytes and CD3−CD4-depleted lung cells. Flow cytometry based cytotoxicity assays have previously been shown to correlate well with the standard ^{51}Chromium release assay [41,42]. Furthermore, the advantage of a flow cytometry based assay is that this assay is not radioactive and multi-color flow cytometry allows the analysis of cell-specific parameters.

Based on the screening of embryonic and adult splenocytes, staining of five candidate NK markers and the 28-4 mAb was combined with the measurement of CD107 expression. Three markers (7C1, 21E3 and 17B12) showed increased CD107 expression within the marker positive subset upon PMA/Ionomycin stimulation, suggesting that these markers recognize chicken NK-cells. Interestingly, 20E5 and 5C7, two markers with the highest expression based on Fig. 2C do not show an increase in CD107 expression in marker positive cells upon stimulation. This confirms the earlier observation that these markers may be expressed to some extend on NK-cells, but are readily expressed by other cells as well. Furthermore, 28-4+ cells in spleen do not show NK-cell function. Interestingly, 28-4+ IEL have been reported to induce lysis of NK-sensitive targets [26]. This suggests that 28-4+ IEL are different from 28-4+ splenocytes which are studied here.

Analysis of CD107 expression in lung cells from IBV infected chickens showed increased CD107 expression compared to lung cells from uninfected chickens. Interestingly, this difference was only observed for CD3−CD8α− cells, which again suggests that activation of chicken NK-cells is paralleled by down regulation of CD8α. These data show for the first time that the ChCD107 assay can be readily used to study chicken NK-cell activation *ex vivo*. Therefore, this assay is a valuable tool to study NK-cell biology in healthy and diseased chickens.

In conclusion, we identified five new markers (7C1, 21E3, 17B12, 20E5 and 5C7) that recognize chicken NK-cells and developed two assays to measure NK-cell activation and cytotoxicity. Although the antigens that are recognized by the different markers are not yet known, the experiments performed in this study suggest that the markers may recognize different populations of NK-cells. It is possible that the frequencies of NK-cells recognized by the different markers may vary between different organs, similar to the results with the 28-4 mAb. These results will lead to a better understanding of NK-cell frequencies and distribution in healthy and diseased chickens.

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References

[1] Trinchieri G. Biology of natural killer cells. Adv Immunol 1989;47:187–376.
[2] Lanier LL. NK cell recognition. Annu Rev Immunol 2005;23:225–74.
[3] Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature 1986;319:675–8.
[4] Ljunggren HG, Karre K. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. J Exp Med 1985;162:1745–59.
[5] Moretta L, Bottino C, Pende D, Vitale M, Mingari MC, Moretta A. Different checkpoints in human NK-cell activation. Trends Immunol 2004;25:670–6.
[6] Cooper MA, Fehniger TA, Fuchs A, Colonna M, Caligiuri MA. NK cell and DC interactions. Trends Immunol 2004;25:47–52.
[7] Moretta A. Natural killer cells and dendritic cells: rendezvous in abused tissues. Nat Rev Immunol 2002;2:957–64.
[8] Nandakumar S, Woolard SN, Yuan D, Rouse BT, Kumaraguru U. Natural killer cells as novel helpers in anti-herpes simplex virus immune response. J Virol 2008;82:10820–31.
[9] Lanier LL, Testi R, Bindl J, Phillips JH. Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. J Exp Med 1989;169:2233–8.

[10] Ritz J, Schmidt RE, Michon J, Hercend T, Schlossman SF. Characterization of functional surface structures on human natural killer cells. Adv Immunol 1988;42:181–211.
[11] Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol 2001;22:633–40.
[12] Ryan JC, Turck J, Niemi EC, Yokoyama WM, Seaman WE. Molecular cloning of the NK1.1 antigen, a member of the NKR-P1 family of natural killer cell activation molecules. J Immunol 1992;149:1631–5.
[13] Arase H, Saito T, Phillips JH, Lanier LL. Cutting edge: the mouse NK cell-associated antigen recognized by DX5 monoclonal antibody is CD49b (alpha 2 integrin, very late antigen-2). J Immunol 2001;167:1141–4.
[14] Hayakawa Y, Smyth MJ. CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. J Immunol 2006;176:1517–24.
[15] Evans DL, Jaso-Friedmann L. Natural killer (NK) cells in domestic animals: phenotype, target cell specificity and cytokine regulation. Vet Res Commun 1993;17:429–47.
[16] Goff WL, Johnson WC, Horn RH, Barrington GM, Knowles DP. The innate immune response in calves to Boophilus microplus tick transmitted Babesia bovis involves type-1 cytokine induction and NK-like cells in the spleen. Parasite Immunol 2003;25:185–8.
[17] Endsley JJ, Endsley MA, Estes DM. Bovine natural killer cells acquire cytotoxic/effector activity following activation with IL-12/15 and reduce Mycobacterium bovis BCG in infected macrophages. J Leukoc Biol 2006;79:71–9.
[18] Sivori S, Pende D, Bottino C, Marcenaro E, Pessino A, Biassoni R, et al. NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. Eur J Immunol 1999;29:1656–66.
[19] Walzer T, Blery M, Chaix J, Fuseri N, Chasson L, Robbins SH, et al. Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. Proc Natl Acad Sci USA 2007;104:3384–9.
[20] Pessino A, Sivori S, Bottino C, Malaspina A, Morelli L, Moretta L, et al. Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. J Exp Med 1998;188:953–60.
[21] Biassoni R, Pessino A, Bottino C, Pende D, Moretta L, Moretta A. The murine homologue of the human NKp46, a triggering receptor involved in the induction of natural cytotoxicity. Eur J Immunol 1999;29:1014–20.
[22] De MA, Biassoni R, Fogli M, Rizzi M, Cantoni C, Costa P, et al. Identification, molecular cloning and functional characterization of NKp46 and NKp30 natural cytotoxicity receptors in Macaca fascicularis NK cells. Eur J Immunol 2001;31:3546–56.
[23] Falco M, Cantoni C, Bottino C, Moretta A, Biassoni R. Identification of the rat homologue of the human NKp46 triggering receptor. Immunol Lett 1999;68:411–4.
[24] Storset AK, Slettedal IO, Williams JL, Law A, Dissen E. Natural killer cell receptors in cattle: a bovine killer cell immunoglobulin-like receptor multigene family contains members with divergent signaling motifs. Eur J Immunol 2003;33:980–90.
[25] Gobel TW, Chen CL, Shrimpf J, Grossi CE, Bernot A, Bucy RP, et al. Characterization of avian natural killer cells and their intracellular CD3 protein complex. Eur J Immunol 1994;25:1685–91.
[26] Gobel TW, Kaspers B, Stangassinger M. NK and T cells constitute two major, functionally distinct intestinal epithelial lymphocyte subsets in the chicken. Int Immunol 2001;13:757–62.
[27] Lillehoj HS. Intestinal intraepithelial and splenic natural killer cell responses to eimerian infections in inbred chickens. Infect Immun 1989;57: 1879–84.
[28] Myers TJ, Schat KA. Natural killer cell activity of chicken intraepithelial leukocytes against rotavirus-infected target cells. Vet Immunol Immunopathol 1990;26:157–70.
[29] Dennis Jr G, Kubagawa H, Cooper MD. Paired Ig-like receptor homologs in birds and mammals share a common ancestor with mammalian Fc receptors. Proc Natl Acad Sci USA 2000;97:13245–50.
[30] Viertlboeck BC, Habermann FA, Schmitt R, Groenen MA, Du PL, Gobel TW. The chicken leukocyte receptor complex: a highly diverse multigene family encoding at least six structurally distinct receptor types. J Immunol 2005;175: 385–93.
[31] Gobel TW. Isolation and analysis of natural killer cells in chickens. Methods Mol Biol 2000;121:337–45.
[32] Sharma JM, Okazaki W. Natural killer cell activity in chickens: target cell analysis and effect of antithymocyte serum on effector cells. Infect Immun 1981;31:1078–85.
[33] Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M, et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods 2003;281. pp. 281: 65–78.
[34] Penack O, Gentilini C, Fischer L, Asemissen AM, Scheibenbogen C, Thiel E, et al. CD56dimCD16neg cells are responsible for natural cytotoxicity against tumor targets. Leukemia 2005;19:835–40.
[35] Lanier LL, Le AM, Ding A, Evans EL, Krensky AM, et al. Expression of Leu-19 (NKH-1) antigen on IL 2-dependent cytotoxic and non-cytotoxic T cell lines. J Immunol 1987;138:2019–23.
[36] Lanier LL. Back to the future-defining NK cells and T cells. Eur J Immunol 2007;37:1424–6.
[37] Johnson WC, Bastos RG, Davis WC, Goff WL. Bovine WC1(−) gammadeltaT cells incubated with IL-15 express the natural cytotoxicity receptor CD335 (NKp46) and produce IFN-gamma in response to exogenous IL-12 and IL-18. Dev Comp Immunol 2008;32:1002–10.
[38] Chen CH, Six A, Kubota T, Tsuji S, Kong FK, et al. T cell receptors and T cell development. Curr Top Microbiol Immunol 1996;212:37–53.
[39] Viertlboeck BC, Wortmann A, Schmitt R, Plachy J, Gobel TW. Chicken C-type lectin-like receptor B-NK, expressed on NK and T cell subsets, binds to a ligand on activated splenocytes. Mol Immunol 2008;45:1398–404.
[40] Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. J Immunol Methods 2004;294:15–22.
[41] Radosevic K, Garritsen HS, van Graft M, de Grooth BG, Greve J. A simple and sensitive flow cytometric assay for the determination of the cytotoxic activity of human natural killer cells. J Immunol Methods 1990;135:81–9.
[42] Goldberg JE, Sherwood SW, Clayberger C. A novel method for measuring CTL and NK cell-mediated cytotoxicity using annexin V and two-color flow cytometry. J Immunol Methods 1999;224:1–9.