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Analysis of chicken intestinal natural killer cells, a major IEL subset during embryonic and early life

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ABSTRACT

Restrictions on antimicrobials demand alternative strategies to improve broiler health, such as supplying feed additives which stimulate innate immune cells like natural killer (NK) cells. The main objective of this study was to characterize intestinal NK cells in broiler chickens during embryonic and early life and compare these to NK cells in spleen, blood and bone marrow. Also T-cell subsets were determined. The majority of intestinal NK cells expressed IL-2Rα rather than 20E5 and 5C7, and showed low level of activation. Within intestinal NK cells the activation marker CD107 was mostly expressed on IL-2Rα^{+} cells while in spleen and blood 20E5^{+} NK cells primarily expressed CD107. High percentages of intestinal CD8αα^{+}, CD8αβ^{+} and from 2 weeks onward also gamma delta T cells were found.

Taken together, we observed several intestinal NK subsets in broiler chickens. Differences in NK subsets were mostly observed between organs, rather than differences over time. Targeting these intestinal NK subsets may be a strategy to improve immune-mediated resistance in broiler chickens.

1. Introduction

Restrictions on the use of antimicrobials in poultry production have made search for other strategies to maintain or improve poultry health, such as enhanced immune responsiveness by feed interventions important (Taha-Abdelaziz et al., 2018). The gut-associated immune system provides the interface between body and intestinal content and protects against invasion of pathogens. Gut-associated lymphoid tissue (GALT) is situated throughout the intestinal epithelium and consists of the Peyer's patches, the intraepithelial lymphocytes (IEL) and the lamina propria leukocytes (LPL) underneath the IEL (Casteleyn et al., 2010; Vervelde and Jeurissen, 1993). Between epithelial cells of the villi, the abundantly present IEL preserve the integrity of the epithelium, regulate interactions with microbiota and complement the first line of defense against pathogens (Smith et al., 2013). The IEL population consists of various types of immune cells including innate lymphoid cells, γδ T cells, CD8^{+} T cells and natural killer (NK) cells (Cheroutre et al., 2011; Schat and Wakenell, 1993; Van Kaer and Olivares-Villagomez, 2018).

NK cells share many characteristics with group I innate lymphoid cells; both cells express the transcription factor T-bet and secrete Th1 cell-associated cytokines like IFNγ and TNFα as reviewed in Artis and Spits (2015). These cytokines are involved in killing of infected cells and especially IFNγ plays a role in the induction of subsequent adaptive immune responses (Fuchs et al., 2013). NK cells lyse target cells without prior sensitization and without restriction by major histocompatibility (MHC) antigens. In humans, NK cells exit continuously from the bone marrow into blood and tissues like spleen and intestine, and undergo progressive phenotypic and functional maturation (Bonanni et al., 2019). A phenotypic distinction is made between NK cells with predominant cytotoxic effector functions (CD56^{dim}CD16^{high}, IL2-Rα^{-}) involved in killing of target cells, and a much smaller population of NK cells (CD56^{bright}CD16$^{dim/-}$, IL2-Rα^{+}) that readily proliferates and mainly produces cytokines (Cooper et al., 2001; Lanier et al., 1986; Nagler et al., 1990). The CD56^{bright} NK cells constitutively express the high-affinity

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heterotrimeric IL-2R including the α-chain, whereas CD56^{dim} NK cells express intermediate-affinity IL-2R and upregulate expression of the α-chain only upon activation (Duggan et al., 2018; Leong et al., 2014). Increased IL2-Rα expression on human NK cells correlates with target cell induced cytotoxicity and cytokine production (Rudnicka et al., 2015). Human NK cells mainly reside in the intestine in frequencies of 40% in IEL, and in lower frequencies of 10–20% in blood and secondary lymphoid tissues like spleen (Caligiuri, 2008; Ferlazzo et al., 2004; Leon et al., 2003; Lynch et al., 2006). The predominantly cytotoxic NK cell subset was shown in humans and mice to be circulating (Farag and Caligiuri, 2006; Lanier et al., 1986), whereas the mainly cytokine-producing subset is observed in tissues in close contact with T cells (Cooper et al., 2001; Farag and Caligiuri, 2006).

Although NK cell biology in chickens is less advanced compared to humans and mice, chicken NK cells have been described as a population of cells that lack surface expression of T and B cell-specific surface markers and are able to kill a NK-susceptible target cell line (Göbel et al., 2001). A high percentage of these NK cells was observed in embryonic spleen (Göbel et al., 2001; Jansen et al., 2010; Zhang et al., 2012) and in the IEL of the duodenum (Göbel et al., 2001) of layer chickens. More recent studies have shown the presence of NK cells that are able to degranulate, which is a measure for NK cell activation (Alter et al., 2004), in various tissues including spleen, lung and blood of layer chickens (Jansen et al., 2010, 2013; Vervelde et al., 2013).

Despite the fact that a pan NK marker is still missing for chickens, expression of many markers has been reported on CD3 negative cells that show enhanced CD107 expression, indicating that these markers are expressed on cells with NK cell function. Interestingly, differential expression of these markers in various organs suggests that also in chickens different NK cell subsets exist. For instance, in the duodenum of adult SPF chickens and layer chickens a high percentage of NK cells expressed the marker 28–4 (Göbel et al., 2001; Jahromi et al., 2018), identified as the chicken orthologue of the IL-2Rα chain (Fenzl et al., 2017). In blood, spleen and lung of layer chickens, NK cells differentially expressed 5C7, which is defined as putative CD11b/c (Wu et al., 2010) or CD11d (Deeg et al., 2020), both molecules involved in adhesion, 20E5 and 7C1 (Jansen et al., 2010, 2013). The avian orthologue of CD56, a pan NK marker in humans, was expressed on a small population of NK cells in the lung and embryonic spleen but not in other tissues (Neulen and Göbel, 2012). Moreover, although CD8α was expressed on chicken NK cells (Göbel et al., 2001; Neulen and Göbel, 2012), its expression was shown to be downregulated upon activation (Jansen et al., 2010). Since at the onset of the present study limited data on NK cells in broiler chickens was available, we set out to first investigate presence and function of the various NK cell subtypes in broiler chickens in the absence of pathogenic challenges, in multiple organs in course of time from the embryonal stage ED14 up to 21 days of age. In addition, other IEL like the γδ T cells and CD8$^+$ T cell subsets have not been described in broiler chickens at multiple timepoints during embryonic and early life.

Stimulating intestinal NK cells through feed may be a strategy to improve health of chickens. Feed additives can influence, either directly or indirectly through the microbiota, the intestinal development and immune responses (Ao et al., 2012; Rostami et al., 2015; Taha-Abdelaziz et al., 2018). Probiotics were shown to increase intestinal mRNA levels of toll-like receptors (TLRs) (Bai et al., 2013), which play a key role in innate immunity and are also expressed on NK cells. Small cationic peptide supplementation of broiler chickens was shown to increase mRNA levels of TLRs and type I and II interferons in caecal tissue upon infection with Salmonella (Kogut et al., 2013), including IFNγ which is secreted by NK cells. However, complementation of these results with phenotypic characterization including cellular assays of intestinal cells such as NK cells are needed to assess possibilities of modulating these intestinal NK cells to improve chicken health.

In this study, we set out to analyze various subsets of NK cells in the intestine and and compared these with subsets in spleen, blood and bone marrow of chicken embryos and in young chickens during the first three weeks of life. We used the monoclonal antibodies specific for 28–4, 20E5 and 5C7 to analyze NK cell subsets over time and we assessed activation of NK cells by determining surface expression of CD107. Analysis of CD107 expression within the IL-2Rα$^+$ and 20E5$^+$ NK cell subsets enabled us to investigate whether in chickens also NK cell subsets with varying functions can be observed. Presence of intraepithelial T cells was assessed over time in comparison to T cells in spleen, blood and bone marrow. Characterization of intestinal NK cell subsets will aid in investigating possibilities to modulate NK cells through for instance feed interventions, which may result in strengthening of the innate immune defense well as subsequent adaptive immune responses in young broiler chickens.

2. Materials and methods

2.1. Animals and tissues

Embryonated Ross 308 eggs were obtained from a commercial hatchery. Eggs were placed in a hatcher at the facilities of the department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, the Netherlands. From literature (Göbel et al., 2001) and earlier work of our group (Jansen et al., 2010), we know that spleens of fourteen day-old embryos contain a high quantity of NK cells. Subsequently, embryonic day (ED) 16 and ED18 embryos were analyzed as representative of late embryonic development. At ED 14, 16 and 18, embryos (n = 10) were euthanized and the small intestines, spleens, femurs and tibiae were collected. Samples of either small intestine or spleen were pooled to obtain sufficient cells and homogenized using a 70 μM cell strainer (Beckton Dickinson (BD) Biosciences, NJ, USA) to obtain single cell suspensions. Lymphocytes were isolated by Ficoll-Paque Plus (GE Healthcare, the Netherlands) density gradient centrifugation for 12 min (1700 rpm, 20 °C), washed in phosphate-buffered saline (PBS; Lonza, Switzerland) by centrifugation for 5 min (1300 rpm) and resuspended at 4.0×10^6 cells/ml in 'complete medium' (IMDM supplemented with 8% heat-inactivated FCS (Lonza); 2% heat-inactivated chicken serum, 100 U/ml penicillin/streptomycin and 2 mM glutamax I; Gibco BRL, United Kingdom). Bone marrow was collected as previously described (De Geus et al., 2012) by cutting the edges of femur and tibia bones and flushing the bone marrow with 10 ml complete medium using a 21G needle and a 10 ml syringe (BD Biosciences). Subsequently, bone marrow samples of 10 embryos were pooled, homogenized using a 70 μM cell strainer, centrifuged for 10 min (1200 rpm, 20 °C) and resuspended at 4.0×10^6 cells/ml in complete medium.

For the analyses during three weeks post hatch, 40 chickens were housed in one floor pen lined with wood shavings (2 kg/m^2) and received a commercial broiler feed *ad libitum*. At day 1, fifteen chickens were euthanized and ileum, spleen, bone marrow and blood were collected and tissue samples of three birds were pooled to obtain biological replicates (n = 5) with sufficient cells to perform the analyses. At day 3, 5, 7, 14 and 21, chickens (n = 5) were euthanized and individual tissues were collected. Ileum segments of approximately 10 cm were harvested and flushed with PBS to remove contents. Next, segments were cut in sections of 1 cm^2 and washed again in PBS. Subsequently, IEL were collected by stirring the sections three times at 200 rpm for 15 min at 37 °C in EDTA-medium (HBSS 1× (Gibco BRL) supplemented with 10% heat-inactivated FCS (Lonza); 0.005M EDTA (Sigma-Aldrich, the Netherlands)). Supernatants containing the IEL were collected after each incubation in EDTA-medium and centrifuged for 5 min at 1200 rpm at 20 °C. Cell pellets were then resuspended in PBS, and lymphocytes were isolated by Ficoll-Paque density gradient centrifugation (12 min, 1700 rpm, 20 °C), washed and resuspended in complete medium at 4.0×10^6 cells/ml. Lymphocytes were isolated from spleen as described in embryos, however, with different Ficoll-Paque density gradient centrifugation conditions (20 min, 2200 rpm, 20 °C). Isolation of lymphocytes from bone marrow was done according to the procedure

described above for embryos. Blood, at least 5 ml, was collected in a tube containing 200 μl of heparin (5000 IE/ml, LEO Pharma A/S, Denmark), diluted with an equal volume of PBS, layered on Ficoll-Paque and centrifuged (20 min, 2200 rpm, 20 °C). Subsequently, lymphocytes were harvested, washed and resuspended in complete medium at 4.0×10^6 cells/ml. Unless mentioned otherwise, lymphocytes were stained for flow cytometric analyses directly after isolation.

To localize markers expressed on immune cells *in situ*, immunohistochemical stainings were performed on ileum tissue of chickens from the control group (PBS inoculation) of a different animal experiment (AVD1080020174425). Chickens (n = 4) were euthanized at day 14 and 3 mm cross sections of ileum were frozen in liquid nitrogen and stored at −80 °C.

To analyze CD107 expression within the IL-2Rα$^+$ and 20E5$^+$ NK cell subsets, viably frozen lymphocytes of IEL, spleen and blood from 21-day-old broiler chickens (AVD1080020174425) were thawed and subsequently stained. Pellets of isolated lymphocytes were resuspended in FCS (Lonza) and ice cold complete medium supplemented with 20% DMSO (Sigma-Aldrich) was added dropwise. Subsequently, cells were stored at −140 °C. Frozen cells were rapidly thawed in a 37 °C water bath followed by adding cold complete medium. Next, the cell suspension was centrifuged, the supernatant was discarded and the pellet was resuspended in complete medium and washed again. Viability of the thawed cells was approximately 90%.

Chickens were housed, handled and treated according to approval by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee of Utrecht University (the Netherlands) under registration numbers AVD108002017863 and AVD1080020174425 and all procedures were performed in accordance with all relevant legislation.

2.2. Immunohistochemistry on ileum cryostat sections

Cryostat sections (7 μm) of the ileum, were placed on precoated slides (Superfrost/Plus, Germany) and stored over silica gel. First, slides were fixed for 10 min in pure acetone and air-dried. Sections were then incubated for 1 h at room temperature (RT) in a closed humidified box with previously optimized concentrations of mouse-anti-chicken-CD3 (CT3; IgG1), mouse-anti-chicken-CD4 (CT4; IgG1), mouse-anti-chicken-TCRγδ (TCR-1; IgG1), mouse-anti-chicken-CD25 (IL-2Rα, AV142; IgG1, Bio-Rad Laboratories, CA, USA) and mouse-anti-chicken-20E5 (IgG1, Developmental Studies Hybridoma Bank (DSHB), University of Iowa, IA, USA) diluted in PBS (Lonza) containing 0.5% bovine serum albumin and 0.1% sodium azide (PBA). Unless described otherwise, antibodies were obtained from Southern Biotech, Birmingham, AL, USA. Following washing in PBS, tissue sections were stained using the VECTASTAIN®Elite®ABC HRP Peroxidase, Standard Kit according to the manufacturer's protocol (Vector Laboratories, United Kingdom). Sections were incubated with horse-anti-mouse-IgG-biotin, washed in PBS followed by incubation in ABC solution. Tissue sections were washed and HRP-enzyme reactivity was revealed by adding 0,5 mg 3,3-diaminobenzidine-tetrahydrochloride (DAB; Sigma-Aldrich) per ml Tris buffer (0.05M, pH 7.6) containing 0.05% H_2O_2, and incubation at RT for 10 min. Sections were washed, briefly counterstained with haematoxylin (Sigma-Aldrich) and rinsed with tap water. Finally, tissue sections were mounted in Kaiser's Glycerol/Gelatine (Boom, the Netherlands) and microscopically analyzed to determine types and location of immune cells in IEL and lamina propria of the ileum.

2.3. Phenotypic characterization of lymphocytes by flow cytometry

Lymphocytes of the intestine, spleen, bone marrow and blood were stained with markers that are known to be expressed on NK cells and T cell subsets. Based on prevalence in our pilot experiments, the following markers were selected to classify NK cell subsets: 28–4, recognizing the IL-2Rα chain (Göbel et al., 2001; Jansen et al., 2010), 20E5, with unknown specificity (Göbel et al., 2001; Jansen et al., 2010), and 5C7, recognizing CD11b/c or CD11d (Deeg et al., 2020; Wu et al., 2010). Lymphocytes (5×10^5) were stained with two panels of antibodies specific for the above NK cell markers and anti-CD3 to be able to exclude T cells (Table 1). In addition, lymphocytes were stained using two panels of antibodies specific for markers on γδ T and various T cell subsets (Table 1). Staining with primary and secondary antibodies (Table 1) was performed using previously optimized concentrations of the various antibodies in 50 μl for 20 min at 4 °C in the dark. Next, cells were washed two times in PBS supplemented with 0.5% BSA and 0.005% NaN_3 (PBA). In addition, lymphocytes were stained with a live/dead marker according the instructions of the manufacturer (Zombie Aqua™ Fixable Viability Kit, Biolegend, CA, USA) for 15 min at room temperature (RT) in the dark. After washing in PBA, lymphocytes were resuspended and fixed using 2% paraformaldehyde in PBS (Merck, Germany) for 10 min at RT. Following fixation, cells were washed again and resuspended in 200 μl PBA of which 150 μl was used to assess fluorescence of cells in a FACSCANTO II Flowcytometer (BD Biosciences). Analysis was performed using FlowJo software (Tree star Inc, OR, USA).

2.4. Assessment of NK cell activation in the CD107 assay

The CD107 assay, which determines NK cell activation by enhanced surface expression of CD107 as a result of releasing granules containing perforin and granzymes, was carried out as described previously (Jansen et al., 2010). Briefly, 1×10^6 lymphocytes were incubated in 0.5 ml complete medium for 4 h at 37 °C, 5% CO_2 in the presence of 1 μl/ml Golgistop (BD Biosciences) and 0.5 μl/ml mouse-anti-chicken-CD107-APC. After anti-CD107 staining, lymphocytes were stained with anti-CD3 and anti-CD41/61 mAbs to exclude T cells and thrombocytes from the analyses (Table 1). A second panel was used to analyze CD107 expression within the IL-2Rα$^+$ and 20E5$^+$ NK

Table 1
Flow cytometry staining reagents.

Cell population	Mix	Primary antibody (mouse-anti-chicken)	Clone/ Isotype	Secondary antibody
NK	a	CD3-APC[a]	CT3/IgG1	–
		28-4-UNL[b]	IgG3	Goat-anti-mouse-IgG3-FITC[a]
		20E5-BIOT[b]	IgG1	Streptavidin-PercP[e]
	b	CD3-APC[a]	CT3/IgG1	–
		5C7-BIOT[b]	IgG1	Streptavidin-PercP[e]
T	a	CD45-APC[a]	IgM	–
		TCRγδ-PE[a]	TCR-1/IgG1	–
		BU-1-FITC[a]	AV20/IgG1	–
	b	CD3-FITC[a]	CT3/IgG1	–
		CD4-APC[a]	CT4/IgG1	–
		CD8α-PE[a]	CT8/IgG1	–
		CD8β-BIOT[a]	EP42/IgG2a	Streptavidin-PercP[e]
NK activation	a	CD107-APC[c]	LEP-100 I 5G10/IgG1	–
		CD3-PE[a]	CT3/IgG1	–
		CD41/61-FITC[d]	11C3/IgG1	–
	b	CD107-APC[c]	LEP-100 I 5G10/IgG1	–
		CD3-PE[a]	CT3/IgG1	–
		CD41/61-FITC[d]	11C3/IgG1	–
		28-4-UNL[b]	IgG3	Goat-anti-mouse-IgG3-APC/Cy7[a]
		20E5-BIOT[b]	IgG1	Streptavidin-PercP[e]

Manufacturer.
[a] Southern Biotech.
[b] Purified supernatant of hybridoma provided by Göbel, T.W., Ludwig Maximilians University, Germany.
[c] Developmental Studies Hybridoma Bank (DSHB), University of Iowa, IA, USA.
[d] Serotec, United Kingdom.
[e] BD Biosciences.

cells (Table 1). After incubation, cells were washed and stained as described in 2.3 and analyzed by flow cytometry.

2.5. Statistical analysis

The assumptions for normal distributed data were not met and therefore non-parametric statistical tests were used. Differences in percentages of immune cell subsets in course of time were analyzed using the Kruskal-Wallis tests accompanied with Dunn's multiple comparisons tests. Differences in percentages of immune cell subsets within a tissue and between tissues were analyzed using Friedman's tests with Dunn's multiple comparisons tests. A p-value of <0.05 was considered statistically significant. All statistical analyses were performed using the software program GraphPad Prism 7 (GraphPad Software, CA, USA).

3. Results

3.1. High prevalence of IL-2Rα$^+$ NK cells in the IEL of the ileum

Immunohistochemistry was performed on tissue sections from the intestine of two week-old broiler chickens to localize IL-2Rα$^+$ and 20E5$^+$ cells, CD3$^+$, CD4$^+$ and γδ T cells (Fig. 1). The IL-2Rα$^+$ cells were present in high numbers among the epithelial cells of villi, crypts and glands (IEL) compared to mild to moderate numbers in the lamina propria of the ileum (Fig. 1A). Unfortunately, we were unable to detect 20E5$^+$ cells in the ileum because the antibody did not work in our hands in immunohistochemical staining (data not shown). The presence of CD3$^+$ T cells was observed more in the IEL compared to the lamina propria (Fig. 1B). CD4$^+$ T cells were mainly present in the lamina propria compared to minimal numbers in IEL (Fig. 1C). Moderate numbers of γδ T cells were observed both in IEL and lamina propria (Fig. 1D).

Based on FACS analyses, the presence of various NK cell subsets in the intestine was determined in embryos and chickens of different ages by analyzing the relative number, defined by the percentage, of CD3 negative cells that express IL-2Rα$^+$, 20E5$^+$ and 5C7$^+$ cells, and CD107 as marker for NK cell activation (Fig. 2A). The percentage of intestinal IL-2Rα$^+$ NK cells tended to be increased at day 1 post hatch (60.74 ± 3.2%) compared to ED14 (15.32 ± 5.9%, p = 0.06) and ED18 (19.25 ± 6.5%, p = 0.08), and remained similar throughout aging (Fig. 2B). The percentage of intestinal 20E5$^+$ NK cells was lower at day 1 (3.9 ± 0.76%) compared to ED14 (14.87 ± 1.4%, p = 0.05) and subsequently increased towards day 21 (11.38 ± 1.9%) to levels similar to those in early embryonic life (Fig. 2C). Also, the percentage of intestinal 5C7$^+$ NK cells was lower at day 1 (1.4 ± 0.27%) than at ED14 (7.3 ± 1.4%, p = 0.07) and increased again to 7.2 ± 2.1% at day 21 (Fig. 2D). When comparing these subsets of NK cells in the intestine post hatch, a considerably higher percentage of IL-2Rα$^+$ NK cells was observed compared to 20E5$^+$ NK cells (Fig. 2B–D). The percentage of CD107$^+$ NK cells varied between 7.66 ± 1.7% (day 21) and 13.71 ± 2.3% (day 5) during embryonic phase and first three weeks of age (Fig. 2E). In addition, CD107 expressing cells were predominantly observed in the intestinal IL-2Rα$^+$ NK cell subset (17.3 ± 0.5%) and less CD107 expression was observed in 20E5$^+$ NK cells (6.4 ± 0.4%, Fig. 2F).

3.2. Splenic NK cells predominantly express IL-2Rα while blood and bone marrow-derived NK cells are mostly 20E5$^+$

In addition to intestinal NK cells, the presence of NK cell subsets in spleen, blood and bone marrow was investigated at different ages. The percentage of splenic IL-2Rα$^+$ and 20E5$^+$ NK cells did not change significantly during embryonic and early life (Fig. 3A and B). The percentage of splenic 5C7$^+$ NK cells was lower at day 1 (4.2 ± 0.2%) compared to ED14 (8.5 ± 0.9%, p = 0.02). At day 7 (7.5 ± 0.4%, p = 0.03), the percentage of 5C7$^+$ NK cells in spleen increased again to similar levels as observed in early embryonic life (Fig. 3C). Similar to intestinal NK cells, also the majority of splenic NK cells in young chickens was IL-2Rα$^+$. Percentages of blood-derived IL-2Rα$^+$, 20E5$^+$ and 5C7$^+$ NK cells were similar during the first three weeks of age (Fig. 3D–F). While the percentage of bone marrow-derived IL-2Rα$^+$ NK cells did not change in time (Fig. 3G), the percentage of 20E5$^+$ NK cells

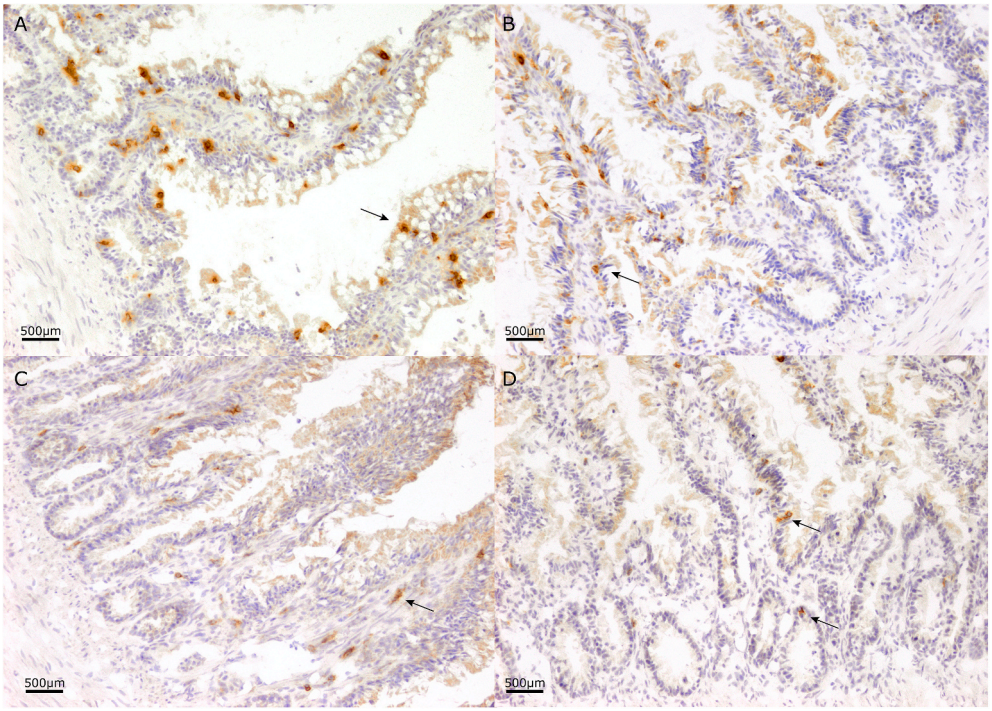

Fig. 1. Localization of cell subsets in IEL using immunohistochemistry. Sections of ileum were stained with IL-2Rα (A), CD3 (B), CD4 (C) and TCRγδ (D) and subsequently counterstained with haematoxylin. Immune cells were microscopically analyzed for their localization in the IEL population or in the lamina propria, stained cells are depicted by arrows. Representative images of a two week-old broiler chicken in a 20× magnification, scale bar = 500 µm.

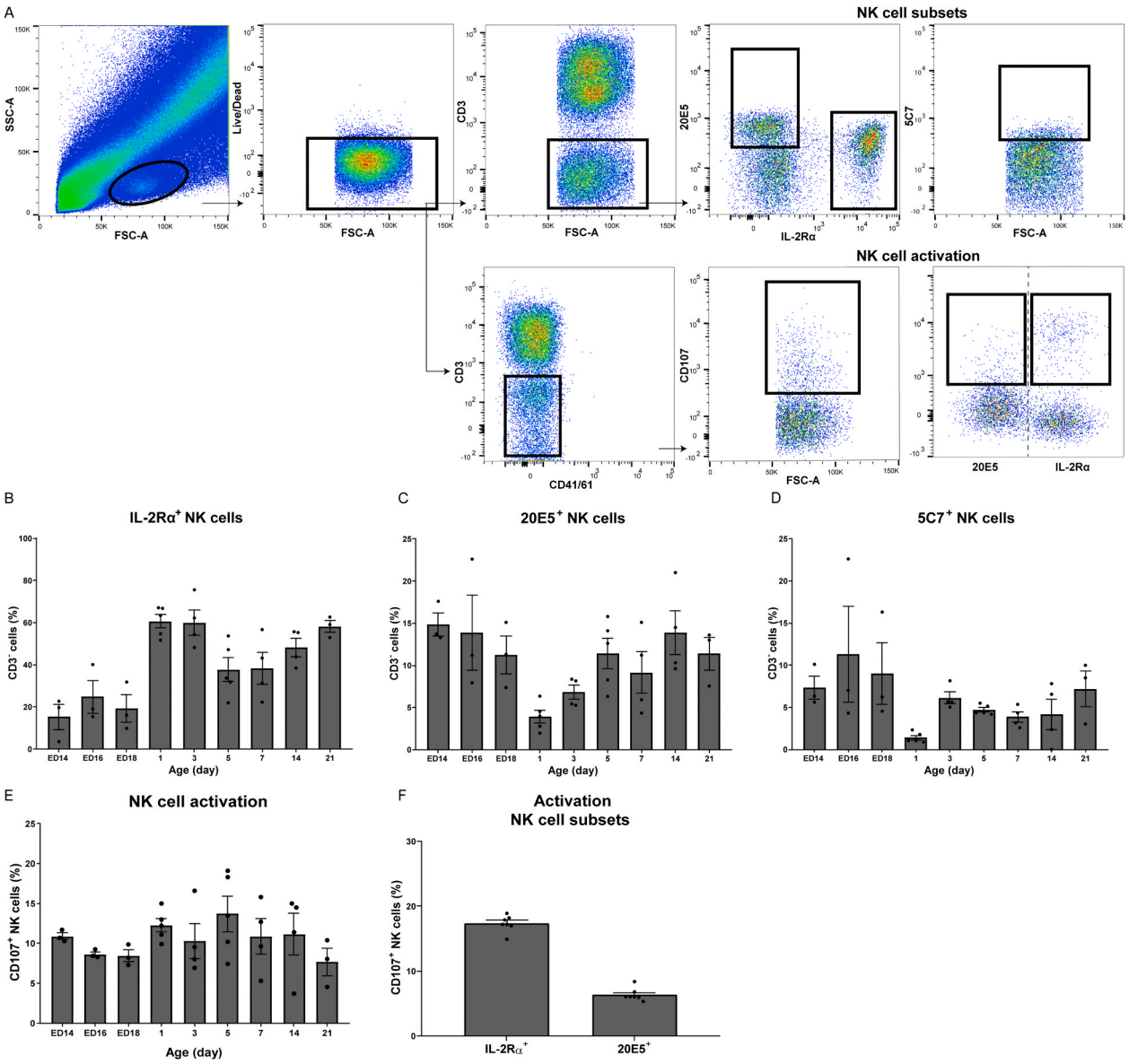

Fig. 2. Phenotypic characterization of intestinal NK cells in embryos and chickens. Gating strategy after isolation of lymphocytes from IEL to analyze NK cell subsets and their activation; lymphocytes were gated, within lymphocytes the live lymphocytes, therein the CD3 negative cells followed by selection of cells expressing IL-2Rα, 20E5 and 5C7 (NK cell subsets) or the CD3 and CD41/61 negative cells followed by selection of cells expressing CD107 in total NK cells and within the IL-2Rα$^+$ and 20E5$^+$ NK cells (A). Percentage of CD3 negative cells (mean ± SEM) that express surface markers IL-2Rα (B), 20E5 (C) and 5C7 (D). NK cell activation was assessed by measuring the surface marker CD107 in total NK cells (E) and in NK cell subsets (F). In figures B–E for embryos n = 3, chickens n = 5, and in F two independent experiments with in total 7 replicates.

increased post hatch from day 1 (2.7 ± 0.3%) to day 7 (16.4 ± 1.9%, p = 0.01) and 14 (17.8 ± 1.9%, p < 0.01) to levels comparable to those in embryonic life (Fig. 3H). Bone marrow-derived 5C7$^+$ NK cells were lower at day 1 (3.0 ± 0.2%) compared to ED18 (7.1 ± 2.1%, p = 0.04, Fig. 3). In contrast to findings in intestine and spleen, most blood and bone marrow-derived NK cells where 20E5$^+$ (Fig. 3D–I).

No significant differences in activation of splenic NK cells was observed upon hatch (Fig. 4A). The percentage of blood-derived CD107$^+$ NK cells tended to increase between day 7 (5.3 ± 1.2%, Figs. 4C) and 14 (19.4 ± 5.0%, p = 0.07). Percentages of bone marrow-derived CD107$^+$ NK cells tended to be lower at day 1 (7.3 ± 0.3%) compared to ED14 (16.0 ± 1.2%, p = 0.09) and significantly decreased at day 7 (5.1 ± 0.2%) compared to ED14 (p < 0.01), ED16 (p = 0.02) and ED18 (p = 0.02), which was followed with a tendency to increase again (Fig. 4E). A higher percentage of CD107$^+$ cells was observed within the 20E5$^+$ NK cell subset compared to IL-2Rα$^+$ NK cells in spleen and blood, the opposite of what was observed in intestine (Fig. 4B, D).

3.3. *High prevalence of γδ T cells, CD8αα$^+$ and CD8αβ$^+$ T cells in IEL of the ileum in young broiler chickens*

Next, the presence of T cell subsets amongst the IEL was determined during embryonic development and in chickens during the first three weeks post hatch (Fig. 5A). The presence of total CD3$^+$ T cells in IEL increased during aging to approximately 70% (data not shown). No CD4$^+$ intestinal T cells were detected in embryos, whereas in young chickens a low presence was found up to day 21 (data not shown). Percentages of γδ T cells in the IEL remained similar upon hatch, but increased from day 14 on to 37.9 ± 5.8% at day 21 (Fig. 5B). Although not significant, a gradual increase was also observed in the percentages of intestinal CD8αα$^+$ T cells upon hatch in ED18 (18.6 ± 8.7%) versus 32.4 ± 4.5% in chickens of 21 days (Fig. 5C). The percentages of CD8αβ$^+$

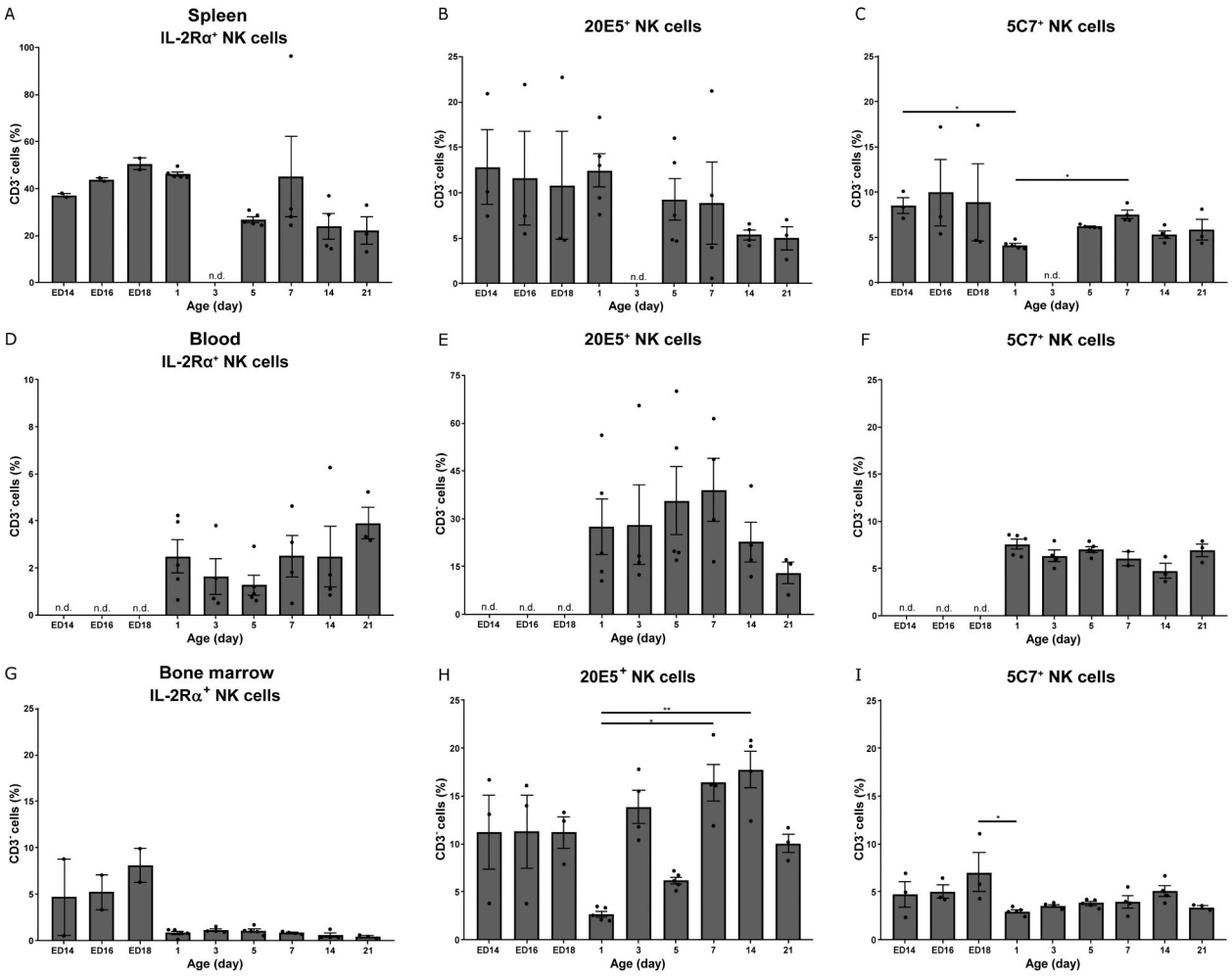

Fig. 3. Phenotypic characterization of NK cells in spleen, blood and bone marrow of embryos and chickens. Gating strategy is similar to IEL. Percentage of CD3 negative cells (mean ± SEM) that express surface markers IL-2Rα, 20E5 and 5C7 in spleen (A–C), blood (D–F) and bone marrow (G–I). In figures A–I for embryos n = 3, chickens n = 5, n. d. means not determined. Statistical significance between ages is indicated as * $p < 0.05$ and ** $p < 0.01$.

T cells increased significantly upon hatch from 1.0 ± 0.2% at ED14 to 49.2 ± 3.7% in chickens of 14 days (p = 0.02, Fig. 5C). No significant differences were found between the percentage of intestinal γδ T cells, CD8αα$^+$ and CD8αβ$^+$ T cells during embryonic development between ED14 and ED18 (Fig. 5B and C). In chickens younger than 7 days, more CD8αα$^+$ and CD8αβ$^+$ T cells were observed compared to γδ T cells (Fig. 5B and C). However, presence of intestinal γδ T increased to levels similar to those of CD8αα$^+$ and CD8αβ$^+$ T cells from day 14 onwards (Fig. 5B and C).

3.4. *Highest prevalence of CD8αβ$^+$ T cells in spleen, blood and bone marrow of young broiler chickens*

Next, T cell subsets in spleen, blood and bone marrow were analyzed in broiler chickens of different ages. The percentage of CD3$^+$ T cells in spleen (54%) and blood (75%) increased during aging similar to what was observed in IEL (70%), whereas in bone marrow a low presence of approximately 2% was found throughout aging (data not shown). An increase was observed in percentages of γδ T cells upon hatch in spleen (Fig. 6A), however, no differences were found in percentages of γδ T cells in blood (Fig. 6C) and bone marrow (Fig. 6E). Furthermore, a higher percentage of CD8αα$^+$ T cells was found in spleen (Fig. 6B) and bone marrow (Fig. 6F) of embryos compared to young chickens, whereas the percentage of CD8αα$^+$ T cells in blood did not change upon hatch (Fig. 6D). Percentages of CD8αβ$^+$ T cells were higher in spleen, blood and bone marrow of young chickens versus embryos (Fig. 6B, D, F).

In embryonic spleen and bone marrow, the majority of the T cells expressed CD8αα$^+$ or CD8αβ$^+$ (Fig. 6). In young chickens, the percentage splenic CD8αβ$^+$ T cells was higher compared to the percentage γδ T cells at day 14 (p = 0.01) and 21 (p = 0.03, Fig. 6A and B). In blood of young chickens, percentages of γδ T cells and CD8$^+$ T cells were similar until two weeks of age, whereas at day 21 the CD8αβ$^+$ T cell subset showed highest presence (Fig. 6C and D). In young chickens, no γδ T cells were found in bone marrow and highest percentages were found of CD8αβ$^+$ T cells from two weeks onwards (Fig. 6E and F).

4. Discussion

The present study was designed to get insight in phenotypical and functional characteristics of subsets of NK cells in general, and intestinal NK cells more specifically, in broiler chickens in course of time during embryonic development and the first three weeks of life.

The majority of intestinal NK cells in the ileum was located in the IEL and expressed the IL-2Rα. Their relative presence, expressed as percentage of total CD3 negative cells, increased post hatch. The high percentage of intestinal IL-2Rα$^+$ NK cells is in agreement with earlier studies in layer chickens and SPF chickens that showed similar percentages at similar ages (Göbel et al., 2001; Jahromi et al., 2018). Also 20E5$^+$ and 5C7$^+$ intestinal NK cells were readily detected with no major changes in presence in the course of embryonic stages and the first

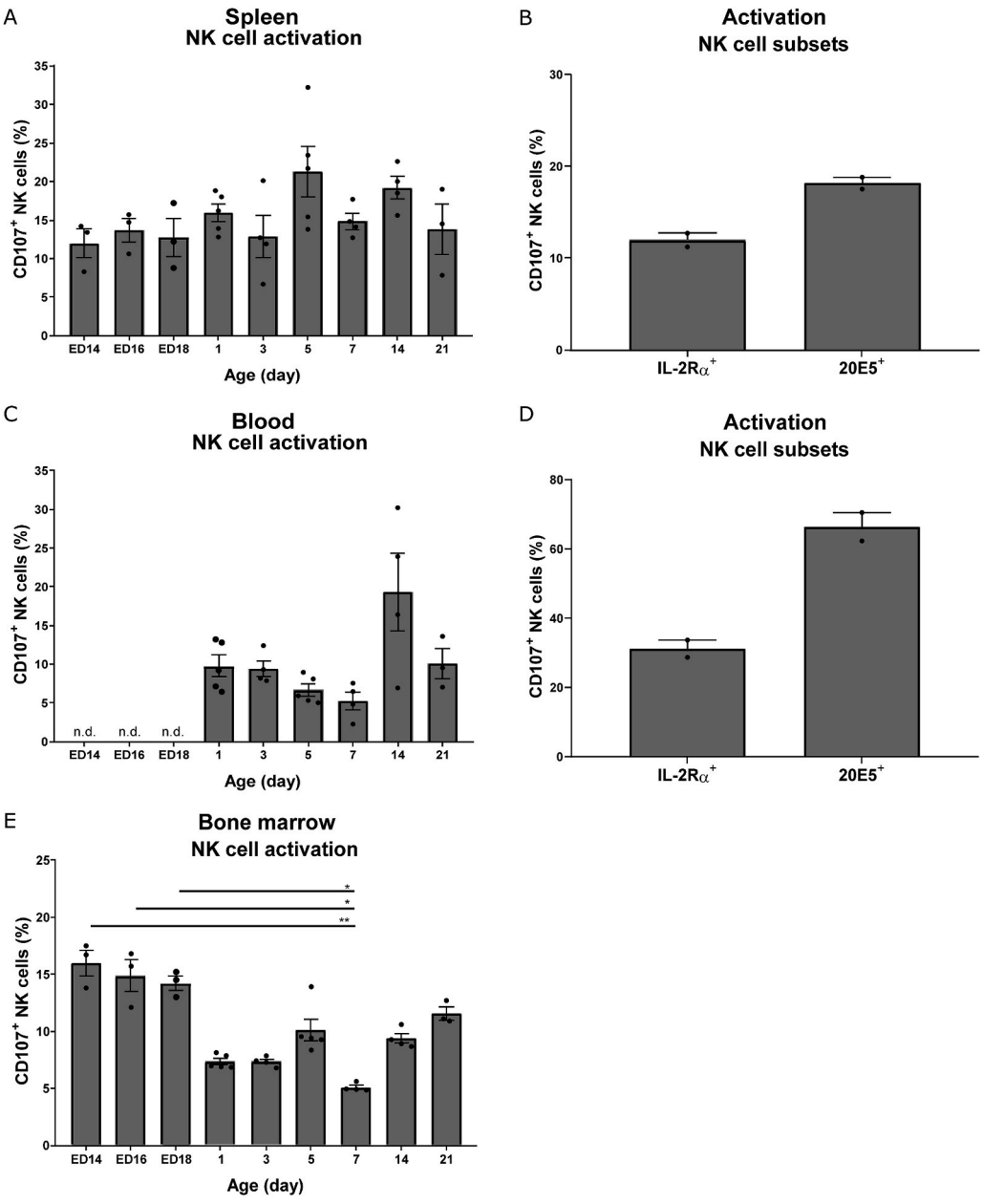

Fig. 4. Phenotypic characterization of activated NK cells in spleen, blood and bone marrow of embryos and chickens. Gating strategy is similar to IEL. NK cell activation was assessed by measuring the surface marker CD107 in total NK cells of spleen (A), blood (C) and bone marrow (E) and in NK cell subsets of spleen (B) and blood (D). In figures A,C, E for embryos n = 3, chickens n = 5, and in B,D two independent experiments. Statistical significance between ages is indicated as * $p < 0.05$ and ** $p < 0.01$.

weeks of life. It should be noted that the marker 5C7 could not be included in the staining panel with IL-2Rα and 20E5, which means we cannot define 5C7$^+$ NK cells as a distinct subset as 5C7 may be expressed on either IL-2Rα$^+$ and 20E5$^+$ NK cells or both. Intestinal percentages of either 20E5 or 5C7 as single markers were found to a lesser extent compared to IL-2Rα. In addition, 10% of intestinal NK cells expressed CD107 and this expression was mainly observed in the IL-2Rα$^+$ NK subset. One day after hatch, the percentage of NK cells expressing IL-2Rα showed an increase, whereas the 20E5$^+$ NK cells decreased.

In our study, the relative number of IL-2Rα$^+$ NK cells in intestine of embryos was similar to bone marrow, but lower in intestine compared to spleen. However, the percentage of IL-2Rα$^+$ NK cells was highest after hatch in IEL compared to the other tissues. This may indicate trafficking of IL-2Rα$^+$ NK cells in the first week after hatch from spleen to the intestine, which has been shown in adult mice where spleen-derived NK cells were found in all NK-containing organs after intravenous adoptive transfer (Grégoire et al., 2007). Alternatively, local NK cells in the intestine may have up-regulated the IL-2Rα$^+$ expression due to dual stimulation of Fc receptors and IL-12R (Duggan et al., 2018), or combination of the IL-12R, IL15R and IL-18R (Leong et al., 2014), which promotes activation as has been shown for human blood-derived NK cells (Duggan et al., 2018; Leong et al., 2014). Interestingly, we observed that the representation of NK cell subsets within the individual tissues varied, since in both intestine and spleen, the IL-2Rα$^+$ NK cell subset was more abundant whereas in blood and bone marrow, the majority of the NK cells expressed 20E5$^+$. The distinction of these NK cell subsets has not been demonstrated in layer chickens during embryonic life. In four-week-old layer chickens, a higher relative presence of splenic 20E5$^+$ and 5C7$^+$ NK cells was observed compared to IL-2Rα$^+$ NK cells (Jansen et al., 2010). We hypothesized that different tissue distributions of IL-2Rα$^+$ and 20E5$^+$ NK cell subsets may be related to specific functions such as cytotoxic activity, which is measured in humans (Alter et al., 2004) and chickens (Jansen et al., 2010) by enhanced surface expression of CD107.

Comparable percentages of CD107 expression were observed on NK cells in spleen, blood, bone marrow and intestine. The percentage of NK

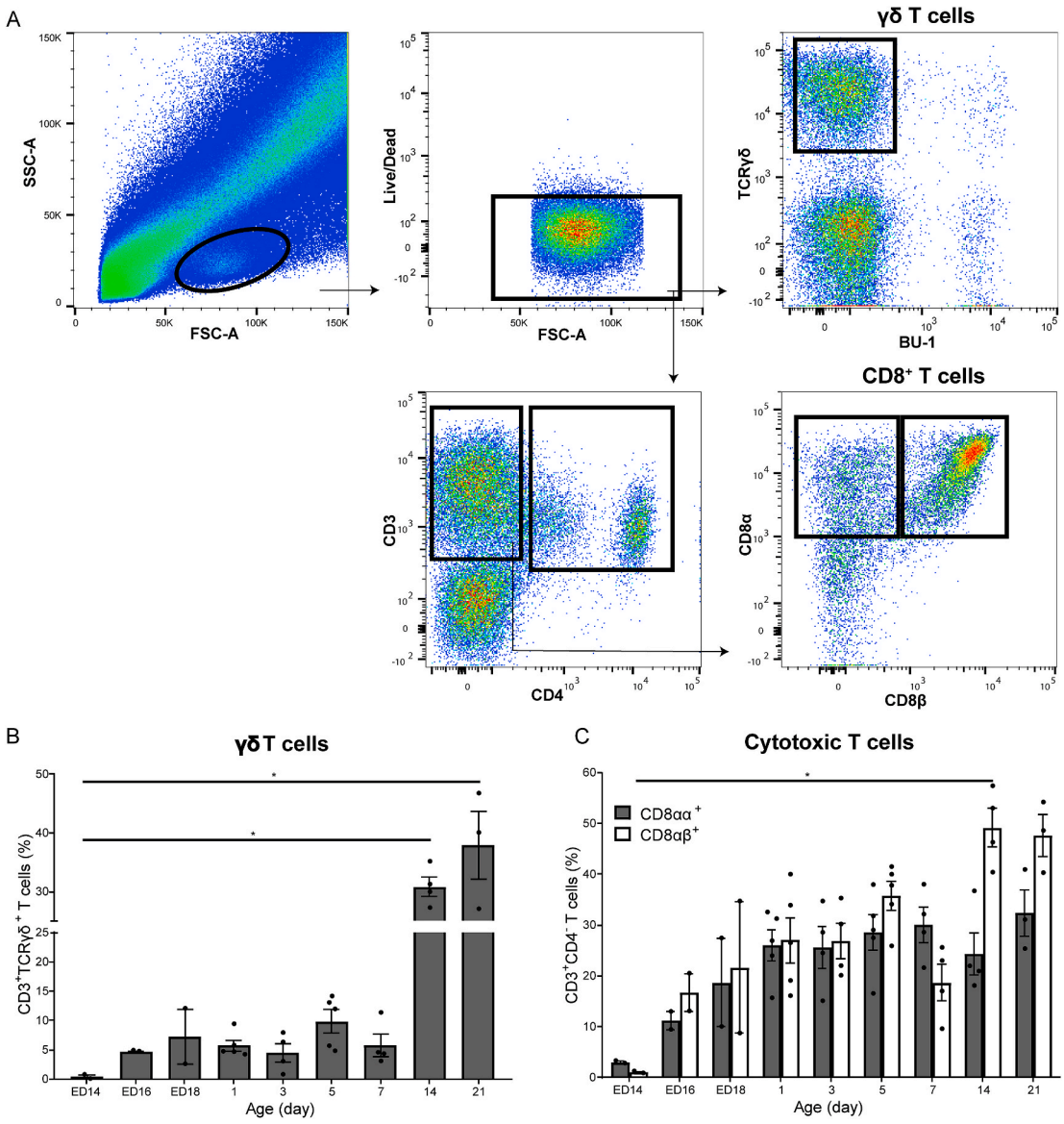

Fig. 5. Phenotypic characterization of intestinal T cells in embryos and chickens. Gating strategy after isolation of lymphocytes from IEL to analyze T cell subsets; lymphocytes were gated, within lymphocytes the live lymphocytes, followed by selection of TCR-1^{+}BU-1^{-} T cells (γδ T cells) or selection within the CD3^{+}CD4^{-} cells of T cells expressing CD8αα and CD8αβ (CD8^{+} T cells) (A). Percentage of live cells (mean ± SEM) that express the surface marker TCRγδ to distinguish γδ T cells (B). Percentage of CD3^{+}CD4^{-} T cells (mean ± SEM) that express CD8αα and CD8αβ to distinguish cytotoxic CD8^{+} T cells (C). In figures B–C for embryos n = 3, chickens n = 5. Statistical significance between ages is indicated as * $p < 0.05$.

cell activation detected in this study was similar to those observed in spleen (Jansen et al., 2010) and blood (Vervelde et al., 2013) of non-infected layer chickens in earlier studies, but lower compared to NK cell activation in infected layer chickens, of which relative presence may increase up to 30% (Vervelde et al., 2013). Interestingly, CD107 expression in spleen and blood was mainly observed on 20E5^{+} NK cells, which is different from what we found in the intestine. Based on these data, both NK cell subsets are implicated in cytotoxic activity, which was different from what we initially hypothesized. Studies with human NK cells have shown that increased IL-2Rα expression was associated with an early stage of NK cell activation (Duggan et al., 2018; Leong et al., 2014; Rudnicka et al., 2015). This may also be the case in chickens, suggesting that the higher percentage of IL-2Rα^{+} NK cells in the intestine and spleen may reflect a population of NK cells in an early stage of activation. The finding that intestinal IL-2Rα^{+} NK cells are involved in cytotoxicity has also been shown in adult layer chickens where IL-2Rα^{+} IEL as putative NK cells showed killing of target cells (Fenzl et al., 2017; Göbel et al., 2001). The activation of intestinal IL-2Rα^{+} NK cells may dependent on local signals, such as interactions with the microbiota either directly through TLRs or indirectly via cytokine production of exposed resident cells (Poggi et al., 2019; Sonnenberg and Artis, 2012). Further research into the IL-2Rα^{+} and 20E5^{+} NK cell subsets is necessary to clarify their functions, which will contribute to the understanding of NK cell biology in chickens. Inducing a higher prevalence of IL-2Rα^{+} NK cells in the intestine, by for instance early life feed interventions, may infer higher protective potency and strengthen the innate immune response during post-hatch development, when adaptive immunity is still immature.

In addition to NK cells, the presence of T cells was also analyzed. In the intestine, the major T cell subsets found in the IEL were γδ- and CD8^{+} cytotoxic T cells, whereas CD4^{+} T cells were mainly present in the lamina propria. This was confirmed with immunohistochemistry data and relative numbers were in agreement with reported levels of intestinal γδ T (Bucy et al., 1988; Tregaskes et al., 1995), CD8^{+} T (Tregaskes

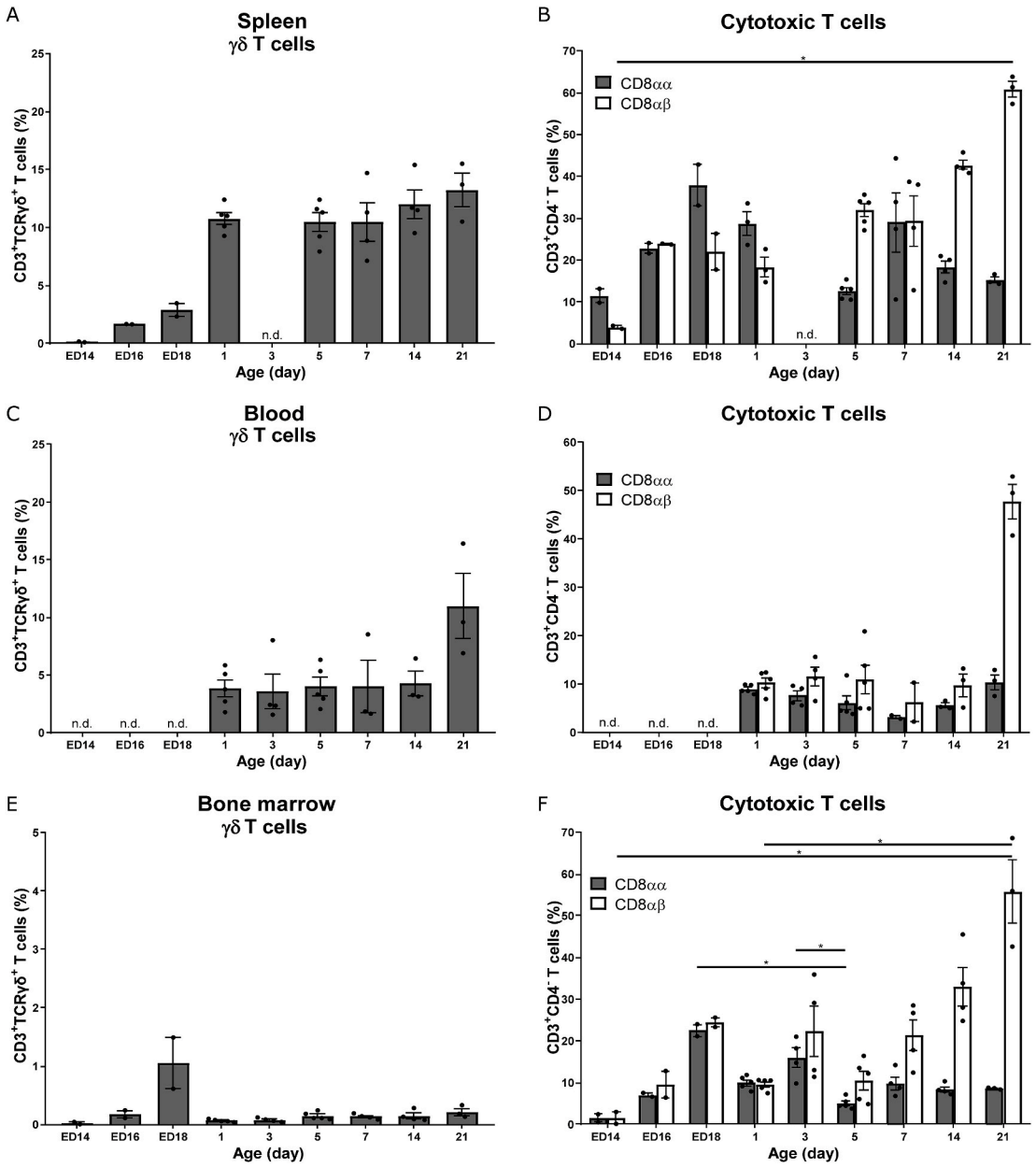

Fig. 6. Phenotypic characterization of T cells in spleen, blood and bone marrow of embryos and chickens. Gating strategy is similar to IEL. Percentage of live cells (mean ± SEM) that express the surface marker TCRγδ to distinguish γδ T cells in spleen (A), blood (C) and bone marrow (E). Percentage of $CD3^{+}CD4^{-}$ T cells (mean ± SEM) that express CD8αα and CD8αβ to distinguish cytotoxic $CD8^{+}$ T cells in spleen (B), blood (D) and bone marrow (F). In figures A–F for embryos n = 3, chickens n = 5. Statistical significance between ages is indicated as * $p < 0.05$.

et al., 1995) and $CD4^{+}$ T cells (Lillehoj and Chung, 1992; Vervelde and Jeurissen, 1993) in layer chickens. From two weeks of age onwards, γδ T cells were more abundant in the IEL compared to spleen, blood and bone marrow and a similar tissue distribution of γδ T cells was observed in layer chickens (Sowder et al., 1988), rodents and humans (Carding and Egan, 2002; Haas et al., 1993). Percentage of intestinal γδ T cells was similar to those found in ruminants (Mackay and Hein, 1989) and pigs (Piriou-Guzylack and Salmon, 2008). Percentages γδ T cells in the blood of broiler chickens were higher compared to humans (Shekhar et al., 2012). This indicates that γδ T cells in chickens are important effector cells at the interface of innate and adaptive immunity in different locations (Fenzl et al., 2017). Within the cytotoxic $CD8^{+}$ T cell population, high percentages $CD8\alpha\alpha^{+}$ T cells were observed in the IEL, whereas in the other tissues high percentages of $CD8\alpha\beta^{+}$ T cells were found. At three weeks of age, percentages of $CD8\alpha\beta^{+}$ T cells were highest in all tissues. Percentages of intestinal cytotoxic $CD8\alpha\alpha^{+}$ and $CD8\alpha\beta^{+}$ T cell subsets were similar to that found in layer chickens (Tregaskes et al., 1995), whereas in mice higher prevalence of $CD8\alpha\alpha^{+}$ T cells was observed (Mayassi and Jabri, 2018). An overview of the data generated in broiler chickens as compared to layer chickens is given in Table S1. Similar to chicken, human T cells predominantly express CD8αβ, however, CD8αα is only expressed on activated T cells, including γδ T and cytotoxic T cells, and forms a small population which significantly expands during chronic infections (Walker et al., 2013). Higher prevalence of $CD8\alpha\alpha^{+}$ T cells was also associated with higher protective immune responses towards viral (Perumbakkam et al., 2016) and bacterial (Pieper et al., 2011) infections in chickens. Another study reported that intestinal $CD8\alpha\alpha^{+}$ T cells showed innate functional characteristics in mice and humans (VanKaer et al., 2014), which might be suggested to be similar in chickens. A higher prevalence of γδ T cells or $CD8\alpha\alpha^{+}$ T cells, which

might be induced by feed interventions, may strengthen the innate response by collaborating with NK cells to improve health of broiler chickens.

In conclusion, we were able to isolate NK cell subsets from IEL and showed that most NK cells expressed IL-2Rα. IL-2Rα$^+$ NK cells are predominantly present in intestine and spleen, while in blood and bone marrow NK cells are mostly 20E5$^+$. Interestingly, the majority of intestinal CD107$^+$ cells is detected in IL-2Rα$^+$ NK cells, whereas in spleen and blood, the majority of CD107$^+$ cells is observed within the 20E5$^+$ NK cell subset. In addition, the IEL showed highest percentages of γδ T and cytotoxic CD8αα$^+$ T cells compared to the other tissues after hatch. The higher prevalence of IL-2Rα$^+$ NK cells in the intestine compared to the other tissues may indicate an early activation stage of intestinal NK cells, which might be caused by local interactions with the microbiota. Higher prevalence of IL-2Rα$^+$ NK cells in the intestine may infer higher protective potency and future studies should investigate whether the presence of this intestinal NK cell subset could be manipulated by early life feed interventions either directly or through the microbiota. This may result in strengthening the first line of defense in broiler chickens during post-hatch development when their adaptive immunity is still immature and may open possibilities to use immune-mediated protection to raise broiler chickens resistant to infectious diseases and reduce antimicrobial use.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2020.103857.

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