Short communication

Rapid NK-cell activation in chicken after infection with infectious bronchitis virus M41

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ABSTRACT

Natural killer (NK) cells are cytotoxic lymphocytes and play an important role in the early defence against viruses. In this study we focussed on NK cell and interferon (IFN) responses after infection with infectious bronchitis virus (IBV). Based on surface expression of CD107+, enhanced activation of lung NK cells was observed at 1 dpi, whereas in blood prolonged NK-cell activation was found. IFN-α and IFN-β mRNA and proteins were not rapidly induced whereas IFN-γ production in lung, measured by Elispot assay, increased over time at 2 and 4 dpi. In contrast, IFN-γ production in blood was highest at 1 dpi and decreased over time down to levels comparable to uninfected birds at 4 dpi. Collectively, infection with IBV-M41 resulted in activation of NK cells in the lung and blood and rapid production of IFN-γ and not IFN-α and IFN-β compared to uninfected birds.

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1. Introduction

Infectious bronchitis virus is a coronavirus that is a major cause of economic losses in the poultry industry and can be involved in respiratory disease, nephritis, and poor egg production and quality. As well as being an economically relevant pathogen in poultry, IBV also bears close resemblance to the human pathogen severe acute respiratory syndrome coronavirus (SARS-CoV; Cavanagh, 2003).

One of the most efficient and rapid host response against viruses consists of the production of type I interferons (IFN-α and IFN-β), an essential part of the antiviral innate immune system (reviewed by Kawai and Akira, 2006). Many if not most nucleated cells are capable of producing type I IFNs, though differences in the production of IFN-α or IFN-β between cells have been described

(reviewed by Thiel and Weber, 2008). Secreted IFNs stimulate adjacent cells to express potent antiviral proteins and stimulate macrophages and natural killer (NK) cells to elicit antiviral responses. IFN-γ, a type II IFN, is predominantly produced by NK, NKT cells, dendritic cells and Th1 CD4+ and CD8+ effector cytotoxic T lymphocytes once antigen specific immunity develops. IFN-γ increases antigen presentation on macrophages and expression of MHC class I molecules, promotes NK cell activity and leukocyte adhesion and binding required for migration. Thus interferon production triggered by the first contact with the viral intruder slows down the virus multiplication, allowing the host's immune system to respond, and helps to establish an adaptive immune response. In a previous study, we showed that IFN-γ production was not only significantly increased in splenocytes of IBV-infected chickens after in vitro restimulation with IBV, but also in splenocytes of IBV-uninfected chickens after 24 h incubation with IBV-M41 (Ariaans et al., 2009). Based on these findings, we investigated whether chicken NK cells are activated upon infection with IBV, which might suggest that this cell population might be the possible producers of the IFN-γ.

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Avian NK cells have been 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 cells express surface CD8αα homodimers, but no T (CD3) or B-cell specific antigens and are able to kill the NK-susceptible cell-line LSCC-RP9 (Göbel et al., 1994). In adult chickens, these cells have been reported in the intestinal epithelial lymphocyte (IEL) population (Göbel et al., 2001). The frequency of avian NK cells in peripheral blood lymphocytes (PBL) and spleen was very low, ranging from 0.5% to 1.0% (Göbel et al., 2001). This is in sharp contrast to NK cell frequencies in mammals, which have approximately around 10% NK cells in blood and in spleen. One explanation for this difference in NK cells frequencies between chickens and mammals can be that the current markers used to detect chicken NK cells are based on NK cell populations in embryonic spleen and the gut and do not necessarily recognize all NK cells in blood and spleen of adult chickens. Recently, we identified additional markers that are expressed on splenic NK cells and developed an assay in which surface expression of CD107 (LAMP-1) is analyzed as a measure NK cell activation (Jansen et al., 2010).

In this study we investigated if NK cells in lung and blood are activated after IBV-M41 infection using an assay based on degranulation (CD107 assay) to study NK cell activation (Jansen et al., 2010), and we measured the production of type I IFNs (IFN-α and IFN-β) and IFN-γ *ex vivo* at mRNA and at protein level using an IFN-α/β reporter assay (Schwarz et al., 2004) and an IFN-γ ELISPOT assay (Ariaans et al., 2008a).

2. Materials and methods

2.1. Chickens and virus

One-day-old SPF layer chickens were housed in isolators. The chickens were given commercially available food according to the manufacturer's instructions. Drinking water was supplied *ad libitum*.

At 5 weeks of age chickens were given 10^5 EID_{50}/ml of IBV-M41 *via* the intratracheal (100 μl) and nasal route (100 μl). Control chickens were given the same volume PBS. At 1, 2 or 4 days post inoculation (dpi) with IBV-M41, 6 birds per time point were euthanized by cervical dislocation and bleeding. PBS inoculated control birds were euthanized at 4 dpi only. Peripheral blood and lung were collected and leukocytes were isolated as described previously by Jansen et al. (2010). Briefly, lung tissue was cut into small pieces and digested in RPMI containing collagenase and DNAse for 30 min at 37 °C and subsequently squeezed through a 70 μm mesh to prepare a single cell suspension. PBMC and lung leukocytes were isolated by density gradient centrifugation using FICOLL-Paque (GE Healthcare).

2.2. Elispot assay

The IFNγ-ELISPOT assay has been described by Ariaans et al. (2008a), minor modifications are described here. Briefly, MultiScreen™-IP 96-well plates (Millipore) were treated with 70% ethanol for 1 min, washed and coated with mouse-anti-chIFN-γ (Biosource), blocked and lung leukocytes or PBMC were seeded at 2×10^5 cells/well in triplicate in culture medium (RPMI-1640, 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin (P/S)). Cells were incubated with culture medium to measure spontaneous release of IFN-γ. The plates were incubated for 24 h at 41 °C, 5% CO_2. IFN-γ was detected by incubation with biotinylated mouse-anti-ChIFN-γ (Biosource) and poly-HRP streptavidin (Sanquin). The assay was developed using TMB substrate (Sanquin) and analyzed using the A·EL·VIS machine and the Eli.Analyse software (Version 4.0) that allows for automated counting of the number of spots based on size and intensity.

2.3. Real-time quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using the RNeasy Mini Kit and DNase treated using the RNase-free DNase Set following manufacturer's instructions (Qiagen Benelux B.V.).

cDNA was generated with reverse transcription using iScript cDNA Synthesis Kit (Biorad). Real-time qRT-PCR was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, AB). Detection of IFN-α, IFN-β, and 28 S mRNA was described by Ariaans et al. (2008b) and detection of a product in the 5'-UTR of the IBV genome was described by Callison et al. (2006). Primers were used at 600 nM and probes at 100 nM concentration. Corrections for variation in RNA preparation and sampling were performed according to Eldaghayes et al. (2006).

2.4. CD107 assay

The CD107 assay has been described previously by Jansen et al. (2010). Briefly, lung cells were resuspended in IMDM medium supplemented with 2% heat inactivated FCS; 8% heat inactivated chicken serum, P/S and 2 mM glutamax at a concentration of 1×10^6 cells/ml. Cells were incubated for 4 h at 37 °C, 5% CO_2 in the presence of 1 μl/ml Golgistop (Becton Dickinson) and anti-chCD107 mAb (mouse-anti Lep-100, DSHB). After incubation, cells were washed in PBS supplemented with 0.5% BSA, stained with mouse anti-CD3 mAb and mouse anti-CD8α (Southern Biotechnology) and flowcytometry was performed. Data were analysed using the software program FlowJO (Threestar Inc.).

2.5. IFNα/β reporter assay

The quail fibroblast cell line CEC-32 carrying a luciferase gene controlled by a short fragment of the chicken Mx promoter was kindly provided by Dr. S. Härtle (LMU, Munich). Cells were seeded at 1×10^5 cells/well and after o/n incubation medium was replaced by serially diluted recombinant IFN (Schwarz et al., 2004), medium control or supernatant from lung leukocytes or PBMC *ex vivo* cultured in medium only for 24 or 48 h. Subsequently, the culture medium was removed, the cells were lysed and luciferase activity was measured using a luciferase assay kit (Promega) as recommended by the manufacturer.
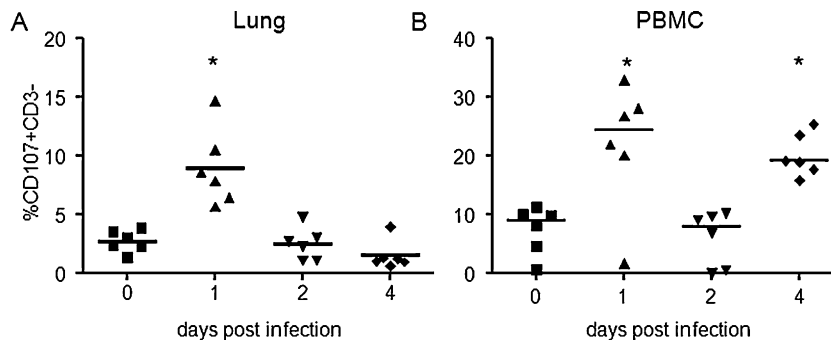
Fig. 1. Enhanced expression of CD107 on CD3− cells in lung and PBMC shows activation of chicken NK cells after IBV-M41 inoculation. %CD107+ CD3− cells in lung (A) or PBMC (B) from uninfected (0 dpi) and IBV infected (1–4 dpi) chickens was analyzed by flowcytometry ($n = 6$ per time point). %CD107+ CD3− cells of individual birds and the mean (bar) are presented with asterisk (*) indicating a significant difference ($p < 0.05$) between uninfected and IBV infected birds.

2.6. Statistical analysis

The assumption of normally distributed data was met and to determine the statistical significance in CD107 expression between the time points a one way ANOVA with a Bonferroni *post hoc* test was used. The assumption of normally distributed data was not met for the Elispot data and Kruskal–Wallis and Mann–Whitney *U* tests were used to compare the statistical differences between time points. Results were considered significant at $p < 0.05$. All statistical analyses were performed using the software program SPSS 20 (SPSS Inc., Illinois).

3. Results and discussion

3.1. IBV induces rapid activation of chicken NK cells in lung and peripheral blood

A subpopulation of cells that can be rapidly activated and recruited upon viral invasion are NK cells. In most farm animals, the definition of NK cells is difficult due to the lack of specific markers (Evans and Jaso-Friedmann, 1993; Boysen and Storset, 2009; Gerner et al., 2009). Chicken NK cells express surface CD8αα homodimers, but lack surface CD3, T cell receptors, and immunoglobulin (Göbel et al., 1994; Zhang et al., 2012). More recently it was shown that upon activation, the CD8αα homodimers were downregulated on the surface of NK cells (Jansen et al., 2010).

In this study, SPF birds were inoculated with IBV-M41 by the nasal–tracheal route and lungs and blood was collected at 0, 1, 2, and 4 dpi. The presence of IBV in the lung at different time points post inoculation was confirmed by qRT-PCR. IBV was detected at 1 and 2 dpi and slightly decreased at 4 dpi (Supplement 1).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2012.11.012.

To measure whether the NK cells were present and activated, a CD107 assay was performed using lung cells of infected and uninfected control birds. Cytotoxic cells contain cytolytic vesicles with proteins like granzyme and perforin. Lining the membrane of these vesicles is CD107 and upon activation the vesicles degranulate and merge with the surface membrane resulting in relocation of CD107 to the surface membrane (Alter et al., 2004). Activation of NK cells was measured by determining the surface expression of CD107 combined with staining for CD3 to discriminate between CD3− NK cells and CD3+ cytotoxic T cells. Upon infection with IBV-M41, CD107 expression was significantly up regulated on CD3− cells at 1 dpi in the lungs (Fig. 1A), suggesting that NK cells were activated. Thereafter, NK cell activity in lung was decreased to background level. In contrast, the percentage activated NK cells in PBMC (Fig. 1B) was much higher compared to the lung and showed a biphasic pattern, with a significant increase at 1 dpi, a decrease to background level at 2 dpi similar to the lung, followed by a significant increase at 4 dpi. The high percentage of activated NK cells in blood might indicate a rapid systemic spread of IBV-M41 (Smith et al., 1985). Transcriptome analysis of pooled tracheas at 3 dpi with an attenuated IBV Massachusetts strain revealed induction of genes involved in cytotoxicity such as Granzyme-A precursor, Fas, CD3 δ and γ chain, MHC II, and TLR2. This expression pattern could be due to cytotoxic T cell responses as suggested by the authors (Wang et al., 2006), but these molecules are also playing a major role in NK-cell cytotoxicity (Zhang et al., 2012). Based on our study a contribution of NK cells at 3 dpi seems very likely and confirms the transcriptome analysis. Furthermore, newly entered but not yet antigen-specific CTLs might be present though not yet cytotoxic, whereas the IBV-M41 specific CTLs can be detected after 9 dpi (Liu et al., 2012).

3.2. IFN-γ but no IFN-α and IFN-β is produced in lung after infection with IBV

The interferon production triggered by the first contact with the viral intruder slows down the virus multiplication, however, many viruses including coronaviruses are capable to cope with and evade the IFN system. Changes in IFN mRNA were measured using qRT-PCR (IFN-α, IFN-β, and IFN-γ), and IFN proteins were measured using an IFN-α/β reporter assay and an IFN-γ Elispot assay. The amount of IFN-α and IFN-β mRNA in the lung of IBV-M41 infected birds did not change during the first 4 days after infection compared to uninfected control birds (Fig. 2A). These findings were confirmed at protein level, no difference in IFN-α/β activity was detected in the supernatants
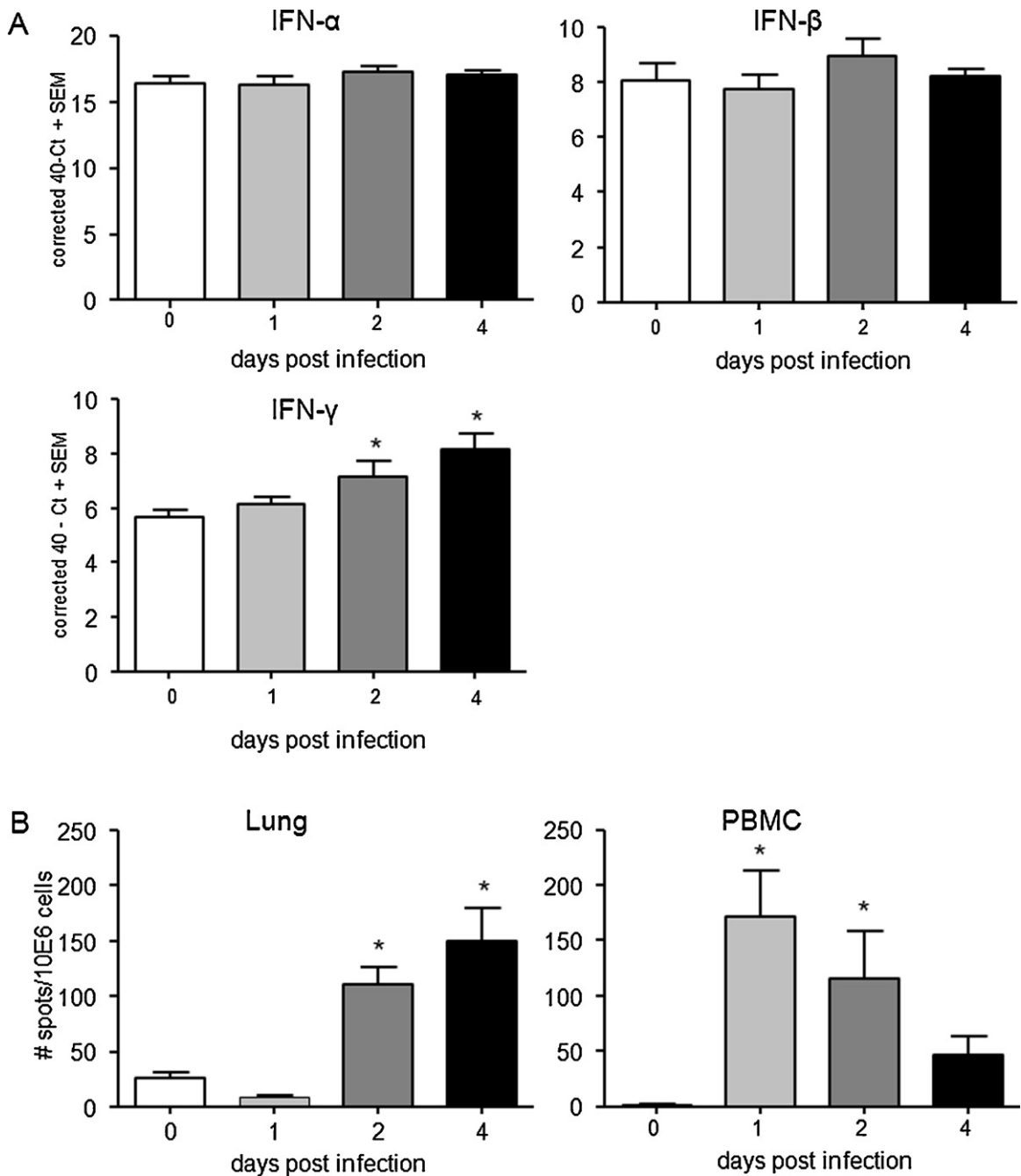
Fig. 2. No increase in type I IFN, but an increase in IFN-γ in lung after IBV-M41 inoculation. (A) Real-time qRT-PCR of mRNA levels normalized to 28S of IFN-α, IFN-β and IFN-γ expressed as 40 Ct (mean of triplicate wells per bird +SEM) in uninfected (white) and IBV infected (gray shades) birds, $n = 6$ per time point. (B) The number of IFN-γ producing cells per 10^6 cells (mean of triplicate wells per bird +SEM) from uninfected (white) and IBV infected (gray shades) was measured using an Elispot assay ($n = 6$ per time point). Cells were isolated from lung and blood at 1–4 dpi and incubated with medium only to measure spontaneous release of IFN-γ due to infection. Asterisk (*) indicates a significant difference ($p < 0.05$) between uninfected and IBV infected birds.

of *ex vivo* lung leukocytes isolated 1–4 days after IBV-M41 infection and cultured without restimulation *in vitro* for 24 h compared to uninfected birds (Supplement 2). The potential of IBV to induce IFN-α/β after infection and its sensitivity to IFN-α/β seems to be IBV strain dependent. IBV-M41 induced low levels of acid stable IFNs in chick embryo and chick kidney cells (CKCs) and no

IFNs after inoculation in embryonated eggs (Otsuki et al., 1979), whereas for example the tissue culture adapted IBV Beaudette strain induced IFNs in embryonated eggs (Otsuki et al., 1979). In contrast, Holmes and Darbyshire (1978) showed that IBV-M41 and IBV-Beaudette failed to yield IFN in primary CKC and in tracheal organ cultures using Semliki Forest Virus inhibition assays. In the latter

study these strains were not susceptible to the inhibitory effects of IFN, whereas Pei et al. (2001) treated CKC with recombinant ChIFN-α which inhibited replication of IBV-Beaudette and Gray strain. Repeated *in vivo* administration of recombinant ChIFN-α before and after inoculation with IBV-Gray strain reduced the clinical respiratory illness.

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In contrast to IFN-α/β, the amount of IFN-γ mRNA in the lung of IBV-M41 infected birds was significantly increased at 2 and 4 dpi compared to the uninfected birds (Fig. 2A). IFN-γ protein production by lung leukocytes also significantly increased at 2 and 4 dpi and coincided with mRNA up regulation (Fig. 2B). In contrast, highest IFN-γ protein production in PBMC was detected at 1 dpi and decreased thereafter. Infection of human PBMC with SARS-coronavirus induced both IFN-α and IFN-γ which peaked within 2 dpi and remained rather stable for 5 days, although individual differences between human subjects were found (Castilletti et al., 2005).

Coronaviruses are capable to cope with and evade the IFN system. It has been described that mouse hepatitis virus (MHV) and SARS-CoV, type 2a and 2b coronaviruses, respectively, can passively escape from getting sensed by cytoplasmic pattern recognition receptors (PRRs). This occurs through formation of double membrane vesicles at perinuclear sites within the cytoplasm where RNA synthesis takes place and actively escape by ORF3b, ORF6, and N protein that are able to inhibit interferon regulatory factor 3 (IRF3; reviewed by Thiel and Weber, 2008). Whether evolutionary conserved IFN antagonists are encoded by all coronaviruses, including IBV, remains to be confirmed.

In future experiments we will investigate whether different IBV pathotypes induce a different activation of the local and systemic NK cells and by performing depletion studies we will investigate if the NK cells are responsible for the rapid secretion of IFN-γ.

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