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Age-dependent differences in cytokine and antibody responses after experimental RSV infection in a bovine model

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Abstract

Respiratory syncytial virus (RSV) causes severe respiratory disease in both infants and calves. As in humans, bovine RSV (BRSV) infections are most severe in the first 6 months of life. In this study, experimental infection with BRSV was performed in calves aged 1–5, 9–16 or 32–37 weeks. Compared to younger animals, older calves showed significantly less fever and lower TNFα levels and less virus-specific IFNγ release. In addition, blood from older animals had more mononuclear cells, more B cells and stronger BRSV-specific IgA and neutralising antibody responses to infection. A strong "inflammatory" but weak humoral antiviral response in very young animals suggests that enhanced inflammation contributes to disease during RSV infection during the early postnatal period.

Keywords: Bovine respiratory syncytial virus; Age-dependent immunity

1. Introduction

Human respiratory syncytial virus (HRSV) is the major viral respiratory tract pathogen of infants and young children and is a pneumovirus in the family Paramyxoviridae, closely related to bovine respiratory syncytial virus (BRSV) [1]. The virus infects respiratory epithelium in both the upper and lower respiratory tract [2]. The antigenic properties of BRSV and HRSV are very similar, and respiratory disease symptoms due to infection with these viruses are comparable in humans and cattle [1]. Natural infections with RSV (both BRSV and HRSV) do not provide complete immunity; the virus can repeatedly reinfect both children and calves, albeit reinfections do give progressively milder symptoms [3].

The frequency of reinfection and the mechanism for evading the immune response is not known. Also, the factors involved in protective immunity to RSV are not known, but both antibody and T-cell responses have been found to be involved [4]. However, reinfection occur even in the presence of high levels of neutralising and complement-fixing serum antibodies [5,6]. The cell-mediated immune response appears to be important for clearance of virus both in the human and bovine system [4,6]. Depletion of cytotoxic T-cells (CD8+) has in studies in both mice [7] and cattle [8] resulted in prolonged extensive replication of virus in the lungs. Also a critical role for CD4+ T-cells in the cellular immune response to respiratory virus infection has been found [9]. The mechanism by which CD4+ T-cells control a virus infection is poorly understood, but is mediated at least in part by IFNγ [10]. In both BRSV [11] and HRSV [12] infections enhanced antigen-specific IFNγ production has been found.

Age seems to be important for development of clinical disease, as most severe HRSV-associated disease is observed in children less than 1 year of age while most severe BRSV-associated disease is observed in calves less than 6 months of age [13]. Epidemiological studies in otherwise healthy children show that infants below 6 months of age have the highest level of RSV hospitalisations [14]; furthermore, premature infants have a highly increased risk of contracting RSV infections [15] and age is therefore considered an important

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risk factor [16]. For calves, various age groups have been assumed to be more prone to develop severe disease, ranging from 0–2 to 4–10 months old calves [17,18]. The conclusion from these studies is that young animals from 1–2 months of age to 6 months of age are most severely affected clinically by BRSV. The underlying causes for the high susceptibility in young individuals are not known, but in infants this increased susceptibility has been proposed to be a consequence of an immature immune system [19,20]. However, studies in infants have shed very little light on the cellular response and antibody response to HRSV, although a disability to produce neutralising antibodies especially against viral envelope glycoprotein in young infants has been shown [21]. Likewise, lower concentrations of immunoglobulin in neonatal than in young calves have been described [22], however, little is known about the immature immune system in cattle, and almost nothing is known about the dynamics of T-lymphocytes in the blood or lymphoid organs. Other possible explanations for the age-dependent variation in susceptibility towards RSV infection include immaturity of the lungs in young individuals, differences in the Th1/Th2 balance, shifts in immunopathology, and non-balanced (exacerbated) inflammatory responses in susceptible young age groups.

Both cotton rats and mice have been used as a model for HRSV infection, however, the disease produced in these rodents differs in a many ways from that in the natural hosts, as reviewed by Domachowske et al. [23], whereas the BRSV infection in calves closely resembles the human RSV infection with similar pathological and epidemiological features [24], and is therefore an ideal model even though compared to rodent studies calves are expensive to maintain and the immunologic resources limited [23] The present study was designed to investigate the influence of age on the clinical course of experimental infection with BRSV, and to study its correlation with the primary specific immune response and cellular and humoral parameters. Calves in three different age groups were experimentally infected with BRSV, the youngest group being 1–5 weeks old, the intermediate group 9–16 weeks and the oldest group 32–37 weeks of age. It was found that the oldest animals with less clinical symptoms had a predominantly humoral immune response, whereas the youngest animals with severe clinical symptoms mounted a predominantly inflammatory and cellular response.

2. Materials and methods

2.1. Animals and experimental infection design

Colostrum-fed 3–9-day-old male Jersey calves were purchased from self sustained herds, reared in isolation units following normal management procedures for calves and experimentally infected as described elsewhere [25]. All but two calves came from the same supplier (IBR- and BVDV-free with low incidence of respiratory disease), the two remaining calves came from another self-sustained herd (also IBR- and BVDV-free with low incidence of respiratory disease), and these additional calves did not differ in clinical response from their group mates. The BRSV inoculum consisted of fourth passage of BRSV isolate 2022 in foetal bovine lung cell (FBL) culture, which had been found free from other viruses, bacteria and *Mycoplasma* spp. All animal were inoculated on the same day, each received $10^{4.6}$–$10^{5.2}$ $TCID_{50}$ by combined aerosol exposure and intratracheal injection. The aerosol inoculum was administered over 10 min through a mask covering nostrils and mouth, while the intratracheal inoculum was suspended in 20 ml phosphate buffered saline and injected into the trachea during provoked heavy inhalation. The inoculum was tested free from other viruses, bacteria and *Mycoplasma* spp. and the calves were free from BVDV and IBR virus.

Three age groups were inoculated: 6 young calves (1–5 weeks old), 6 calves of intermediate age (9–16 weeks old) and 4 old calves (32–37 weeks old). The calves were monitored daily for clinical signs, including rectal temperature and respiratory rate. Serum samples for antibody analysis, and heparin-stabilised blood samples for determination of total white blood cell count and distribution of cellular subsets, were extracted before BRSV inoculation and at days 1, 4, 6, 8, 11, 13, 15, 18, 21 and 28 post inoculation (p.i.).

Two animals died (one young age calf and one intermediate age calf) before day 18 p.i. and were excluded from the analyses. Clinical observation scores included respiratory rate (per minute) and body temperatures recorded between days 3 and 7 p.i. The pathological borderlines were based on previous observations, and were 40/min for respiratory rate and 39.3 °C for body temperature. The score for respiratory reaction and temperature score was calculated by subtracting the borderline values and summarizing the data between days 3 and 7 p.i. (sum: Σ_{3-7} (clinical observation − borderline value) = score) as previously described [25].

2.2. Determination of antibody responses

Serum samples were tested for BRSV specific IgM, IgA, IgG1, IgG2 and for neutralizing antibodies responses by ELISA, as previously described [26]. Sampling at days −7, −3, 1, 4, 6, 8, 9, 11,13,15, 18, 21, 28, 34, 41, 48, 53, 55, 57, 60, 62, 64, 69 and 77. The magnitude of the antibody response was calculated as the area under the response curve from days 8 to 48 p.i.

2.3. Determination of total white blood cell count and distribution of cellular subsets

In blood samples (heparin-stabilised blood) total white blood cell concentrations (WBC/ml) were automatically counted (AutoCounter AC990, SVElab), and distribution of granulocytes, monocytes and lymphocytes was measured by flow cytometry (FACSCan, Becton Dickinson) based on profiles of size and granularity, giving % cell type relative to the total number of white blood cells as the primary

reading. Peripheral blood mononuclear cells (PBMCs) were prepared from buffy coat cells after centrifugation (1200 × *g*, 5 min) of heparin-stabilised blood, followed by buoyant densisty separation on Ficoll-Paque PLUS (Amersham Pharmacia Biotec). PBMCs were collected and resuspended in PBS. For determination of cell subsets, aliquots of approximately 10^6 cells (PBMC) were incubated (30 min, 4 °C) with either mAbs against bovine CD2 (CC42, Serotec), bovine CD4 (CC30, ATTC), bovine CD8 (CC63, ATTC), bovine WC1 on γδ cells (CC15, Serotec), or bovine CD21 on B cells (CC21, Serotec) in optimal dilutions, using PBS as negative control. This was followed by incubation (30 min, 4 °C) with secondary FITC-conjugated anti-mouse immunoglobulin antibody (F0313, DAKO). Washing was performed with PBS containing 0.1% azide and 0.5% BSA. Labelled cells (10.000 gated events) were acquired on FACScan and analysed by use of the software program CellQuest (Becton Dickinson). The total number per ml of mononuclear cells, lymphocytes, granulocytes, and monocytes were computed using the formula:

(WBC count) × (% cell type of WBC)

2.4. In vitro stimulation of PBMC with BRSV antigen

In vitro stimulation for measurement of IFNγ in cell supernatants and for intracellular staining was performed on PBMCs from samples obtained before inoculation of BRSV (day −7 p.i.) and after inoculation at days 11, 13, 18, 21 and 28 p.i. for IFNγ determination in supernatants and at days 11, 13, 21 and 28 p.i. for intracellular staining.

BRSV antigen used for in vitro stimulation, consisted of polyethylenglycol 6000-precipitated BRSV (Danish field strain 88Lu195) [27] from foetal bovine lung cell culture (FBL) infected with BRSV, while the control antigen consisted of FBL without BRSV. BRSV antigen and control antigen was used in a final solution of 1:150.

PBMC were isolated from heparin-stabilised blood as described above and resuspended in RPMI (RPMI medium 1640, Gibco-BRL) with antibiotics (penicillin 100 U and streptomycin 100 μg/ml) and 10% foetal calf serum (FCS, Gibco-BRL). The cell suspension 1 ml of 3×10^6 cells/ml was incubated in 24-well cell-culture plates (Greiner). The incubation for detection of cytokine in the supernatants was performed for 44 h at 37 °C in 5% CO_2 in the presence of *Staphylococcal* enterotoxin B (SEB 5 μg/ml) (ALEXIS Biochemicals, Tx-BP202), BRSV antigen, control antigen or RPMI (4 wells per sample). SEB was chosen as a suitable positive control as it is a superantigen that directly activates most bovine T-cell subtypes by coupling to the TCR elements outside the antigen binding groove without a need for prior internalisation and processing by APC's [28]. This induces a monoclonal T-cell activation mimicking the specific T-cell receptor induced stimulation occurring with BRSV antigen.

Incubations for intracellular staining were performed for 48 h. For the last 4 h of incubation the cells were incubated with monensin (Sigma–Aldrich) at a final concentration of 1.5 μg/ml. Supernatants were harvested after centrifugation of the plates (1500 × *g* for 10 min), and stored in 96-well Masterblock® (Greiner) at −20 °C for later measurements of IFNγ.

2.5. IFNγ ELISA

The measurement of IFNγ in cell culture supernatants was performed using a previously developed bovine IFNγ ELISA [29]. Briefly, 96-well plates (MaxiSorp™, NUNC were coated with 100 μl monoclonal anti bovine IFNγ (clone cc302, Serotec) at 1 μg/ml in PBS and incubated overnight at 4 °C. Then the wells were blocked with PBS containing 1% (w/v) casein (Casein Hammerstein, MW 75,000–100,000; ICN Biomedicals Inc.) and 0.05% Tween 20) for 1 h at 37 °C (blocking buffer). The wells were washed 6 times in PBS with 0.05% Tween 20. Samples (50 μl) were added with an equal amount of blocking buffer and incubated for 2 h at room temperature. Then washing was performed 6 times, followed by incubation with a purified polyclonal rabbit anti-recombinant bovine IFNγ antibody 1:4000 in blocking buffer.

Then horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (ZyMAX, Zymed) at 1:20,000 in blocking buffer was added for 1 h at room temperature. Staining was performed with orthophenyldiamine (Kem-En-Tec) according to the manufacturers instructions. Absorbance was read at a wavelength of 490 nm (reference wavelength 650 nm).

Recombinant IFNγ was used as a standard from the concentration of 20,000–9 pg/ml. Standard curves were used for interpolating unknown concentrations from samples. Cut off value was set to 30 pg/ml.

2.6. Intracellular staining for cytokines

After antigen in vitro stimulation, 100 μl of 20 mM EDTA (Sigma–Aldrich) was added to the cells to give a final solution of 2 mM and incubated for 10 min at room temperature. Cells were harvested and then washed twice in washing-buffer (PBS with 0.5% BSA, 0.1% azid, 1% autologous plasma). Hereafter, a volume of 1 ml lysis-solution (FACS™ lysing solution, Becton Dickinson) was added to each sample and incubated for 10 min at room temperature for initial lysis of remaining erytrocytes and fixation of PBMCs. These fixed cells were then suspended in washing-buffer and kept at 4 °C up to 48 h. Before further use, cells were washed twice in washing buffer and permeabilized by adding droplets by droplets of 2.5 ml of saponin-buffer (PBS, with 1% plasma, 0.1% azide, 0.1% Saponin (Sigma–Aldrich)) to the cell-suspension under constant agitation. Cells were subsequently washed twice in saponin-buffer and then incubated for 60 min at 4 °C with anti-boTNFα (in-house monoclonal antibody, clone 3.28) at 1 μg/ml, anti ovine IL-6 (clone 4B6, Serotec) at 5 μg/ml, anti-porcine IL-10 (clone 945 A, Biosource 945A) at 5 μg/ml, anti-boIFNγ (clone cc302, Serotec) at 5 μg/ml or an isotype control of mixed IgG1 and

IgG2 antibodies (DAK-G01 and DAK-G05, DAKO) each at 5 μg/ml. The cells were then washed twice in saponin-buffer and incubated with a phycoerythrin-conjugated $F(ab')_2$ fragment of a rabbit anti-mouse immunoglobulin (2.5 μg/ml F0439, DAKO) for 30 min at 4 °C. Cells were then washed twice in PBS and finally resuspended in PBS with 1% para-formaldehyde at 4 °C, and later analysed by flow cytometry (FACSCan, Becton Dickinson).

A typical forward- and side-scatter lymphocyte gate was set, and 25,000 gated events within this gate were acquired and analysed by use of the software program CellQuest (Becton Dickinson) for measuring cytokine positive lymphocytes.

Percent cytokine positive cells after specific BRSV antigen stimulation was calculated by subtracting buffer control stimulated signal and the isotype control signal from the specific BRSV antigen signal getting the final corrected percentage of positive cells. We consistently observed that there was no cytokine response whatsoever from the negative control cells ("stimulated" with uninfected cells) and therefore, only the buffer control was subtracted. Gating was done in such a way that buffer control was around 0.2–1% of the cells, isotope control was usually lower. Similar corrections/calculations were performed with the positive control of superantigen (SEB) stimulated cells.

2.7. Statistics

One-way Analysis of variance (ANOVA) with Bonferroni's multiple comparison post test was performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, California, USA).

The ANOVA assumes that the sampled data are from populations that follow a Gaussian distribution, however, it was not possible to guarantee a Gaussian distribution in all sampled data. Therefore, the ANOVA was supplemented by non-parametric statistics (post test; Kruskal–Wallis), which does not rely on this assumption, since it is based on ranking values from low to high. Both statistical tests gave similar results. *P*-values shown in the text are values from the ANOVA test.

3. Results

3.1. Clinical findings

One young calf and one intermediate age calf died during the first weeks after BRSV inoculation and were both excluded from the study. Increased rectal temperature was measured in all inoculated calves; with a significantly higher score (sum of temperature during infection) of body temperature in the group of young calves compared to the group of old calves, as shown in Fig. 1. Likewise, higher respiratory rates were observed in the young age group and intermediate age group compared to the old age group calves (not statistically significant).

3.2. Development of antibodies

Daily serum samples were tested for antibodies. The magnitude of the BRSV-specific IgM, IgA, IgG1, IgG2 and neutralizing antibodies responses are depicted as a summation figure integrating the response of each antibody isotype in each age group from days 8 to 48 p.i. (Fig. 2).

The magnitudes of the maternal IgG1 antibodies (results not shown) in the age groups before inoculation were highest in the youngest age group (mean 45) and lower in the intermediate age group (mean 20.3) and too low to estimate in the oldest age group.

In response to the experimental BRSV infection, all calves in the oldest age group had an IgA response (mean 847.5) while only one out of five calves responded in both the intermediate age group (mean 76) and in the young age group (mean 84), which were statistically significantly different ($P < 0.05$). A similar pattern could be seen for the IgG1 responses (not statistical significant). The IgG2 response was very low, but the highest response was in the two youngest groups (means for young 962, intermediate 230 and old 60). All calves in the three age groups showed an IgM response. In the oldest group, there were two animals out of four that responded with a high IgM response and two animals with a

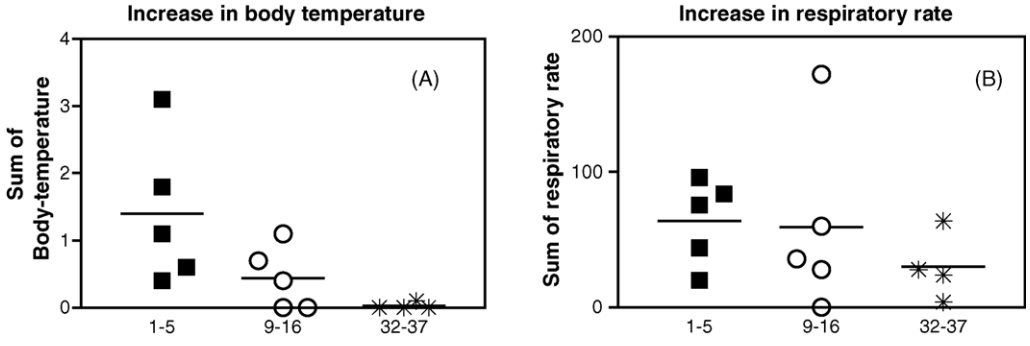

Fig. 1. Age-dependent differences in clinical responses after BRSV infection, shown as the sum of clinical scores as described in Section 2. Means for each age group are shown as horisontal lines. Black squares represent the youngest age group (1–5 weeks old), circles represent the intermediate age group (9–16 weeks old) and the asterisks represent the oldest age group (32–37 weeks old). (A) Sum of body temperature. (B) Sum of respiratory rate.

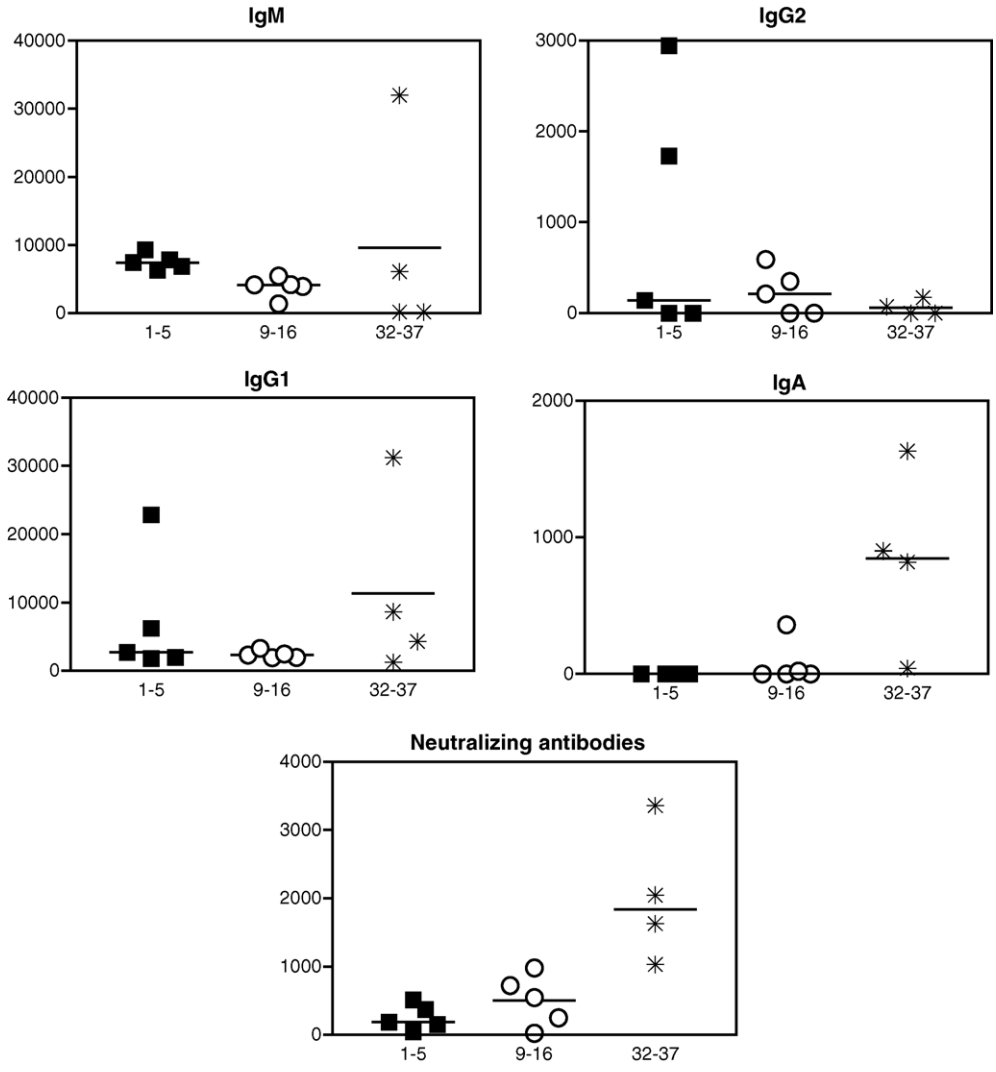

Fig. 2. Age-dependent differences in antibody responses after BRSV infection, shown as the sum of antibody scores as described in Section 2. *Y*-axes show arbitrary units. The median for each age group are shown as horisontal lines. Black squares represent the youngest age group (1–5 weeks old), circles represent the intermediate age group (9–16 weeks old) and the asterisks represent the oldest age group (32–37 weeks old). Note that the *Y*-axis differs for the different antibody types.

low IgM response (appears negative in Fig. 2, but are low), while the group variation was negligible in the intermediate age group and the youngest group. Comparing the intermediate age group (mean 3816) and the youngest age group (mean 7560), the youngest group responded with the strongest IgM response.

No general pattern of high responder/low responder animals could be seen; as individuals with high titers of one isotype, did not necessarily have high titers of other isotypes.

3.3. White blood cell (WBC) count

White blood cell count is shown in Fig. 3. Significant differences ($P < 0.05$) between the WBC count of all the age groups were found throughout the infection except at day 8 p.i. (Fig. 3). Even before BRSV inoculation there was a significant difference ($P < 0.05$) between the age groups in WBC counts, with a mean WBC counts of 9.4×10^6 cells/ml in the oldest age group ($n = 4$) and 8.1×10^6 cells/ml in the intermediate group ($n = 5$) while the youngest group of calves had a significantly lower cell count (mean 6.5×10^6 cells/ml) ($n = 5$).

Alterations in numbers of WBC were observed after inoculation with BRSV, as shown in Fig. 3. Significant differences ($P < 0.05$) between the intermediate age group and the young age group (calves in the oldest animal group were executed at day 28 p.i.) remained in all tested samples up to day 77 p.i. (data not shown).

3.4. WBC distribution

Alterations in WBC are further detailed in Fig. 4, showing first the differentiation of WBC into mononuclear cells and polynuclear cells, and hereafter the differentiation of

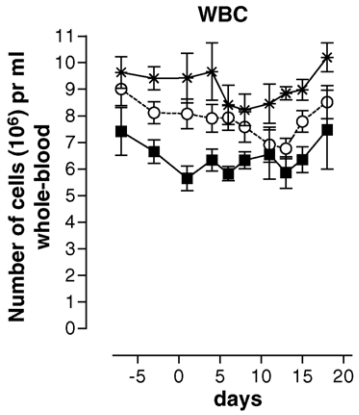

Fig. 3. The kinetics of white blood cells (WBC) in whole blood during BRSV infection. *X*-axis shows days post infection. *Y*-axis is total number of cells per 10^6/ml whole blood. Black squares represent the youngest age group (1–5 weeks old), circles represent the intermediate age group (9–16 weeks old) and the asterisk represents the oldest age group (32–37 weeks old).

mononuclear cells into mononuclear cells in lymphocytes and monocytes. All are shown both as numbers of cells pr. ml whole blood, and as percentage of WBC. The significant difference in WBC between the age groups, with highest number in the oldest groups, was due to differences in the total amount of mononuclear cells per ml (upper row in Fig. 4); which again was almost entirely accounted for by the absolute numbers of lymphocytes per ml. The oldest groups, therefore, had significantly higher numbers of lymphocytes than the younger animals pr. ml both measured in relative and absolute numbers (Fig. 4). However in all groups lymphopenia could be seen at day 6 p.i.

Looking into percentage of subsets it was found that the youngest age group consistently had a higher percentage of polynuclear cells (granulocytes) as shown in (lower row in Fig. 4) throughout the entire infection (mean value), namely a level of 34.8%, which was significantly higher than both the intermediate age group (22.4%) and the oldest age group (22.6%) ($P < 0.0001$).

3.5. Distribution of lymphocyte subpopulations

The distribution of lymphocyte subsets was determined on isolated PBMC fractions. Percentages of lymphocyte subsets according to the three age groups of calves are shown in Fig. 5. Subsets were not calculated as number of cells/ml as the error introduced by multiplication of the percentages (the primary reading) with the absolute number of lymphocytes

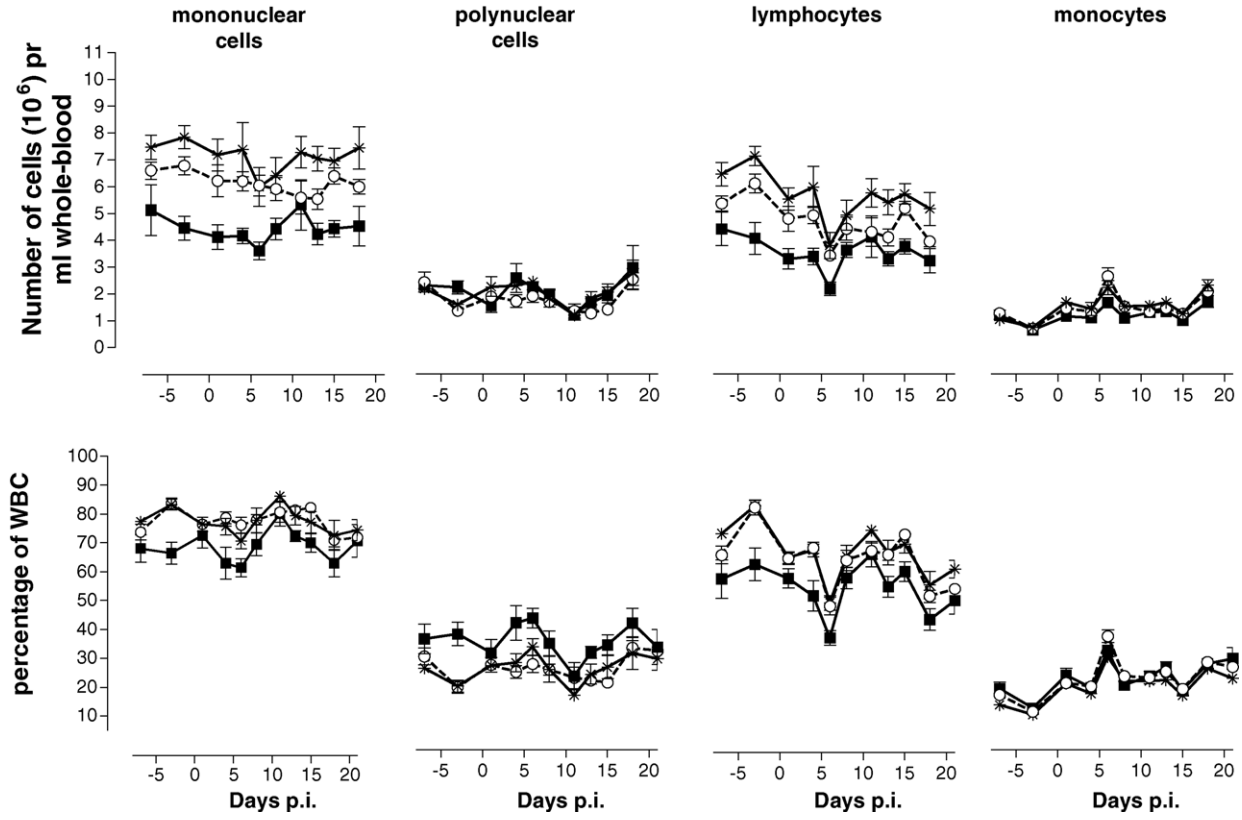

Fig. 4. Age-dependent changes in leukocyte subsets over time in response to experimental infection with BRSV. Upper row is depicted as absolute numbers pr ml whole blood, and lower row depicted as percentage of leukocytes in whole blood. *X*-axis shows days post infection. *Y*-axis shows total counts in number of cells pr ml. Black squares represent the youngest age group (1–5 weeks old), circles represent the intermediate age group (9–16 weeks old) and the asterisks represent the oldest age group (32–37 weeks old).

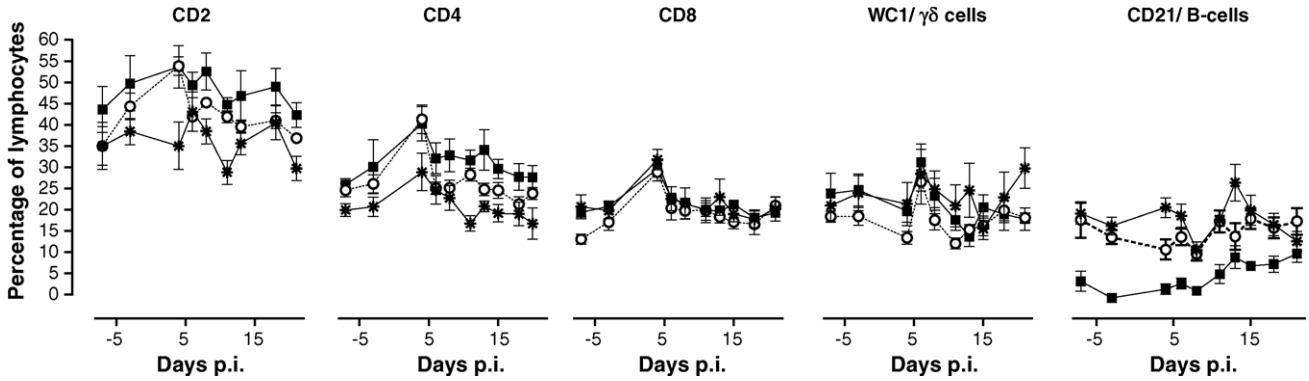

Fig. 5. Age-dependent changes in the percentages of CD2, CD4, CD8, γδ Tcells (WC1) and B-cells (CD21) in PBMC after BRSV infection. *X*-axis shows days post infection. *Y*-axis shows percentage of PBMC in whole blood. Black squares represent the youngest age group (1–5 weeks old), circles represent the intermediate age group (9–16 weeks old) and the asterisks represent the oldest age group (32–37 weeks old).

(see Fig. 4), which in turn was calculated as described above, was considered to be too big.

3.5.1. Age group differences in lymphocyte subset distribution before infection

Before infection (see Fig. 5, days −3 and −7 p.i.) the most pronounced differences in lymphocyte subsets were found in the percentages of CD21 positive B cells, with a much higher percentages of B-cells ($P < 0.001$) in the old and intermediate age groups compared to the young age group. This difference was also found in absolute numbers (cells per ml) (not shown). Before infection (day −3 p.i.) the mean percentage of B cells was 19.9% in the old age group, 17.5% in the intermediate age group, and 4.4% in the young age group. The percentages of CD2+ and CD4+ cells were generally lower in the old animals, and higher in the young animals before infection (day −3 p.i.). However in absolute numbers no significant differences were found (data not shown), but absolute numbers of both CD8 and γδ cells were highest in the oldest animals.

3.5.2. Age group differences in lymphocyte subset distribution after infection

Changes in lymphocyte subsets after BRSV infection were most obvious for the relative number (percentage of mononuclear cells) of CD4+ cells, CD8 + cells and B cells (see Fig. 5). Increases of CD4+ and CD8+ positive cell percentage were detected around days 4–6 p.i. in all age groups compared to pre-infection levels (day −5 p.i.). At days 6–11 p.i., the percentage of CD4+ cells in the oldest age group was significantly lower than the percentage of CD4+ cells in the youngest age group ($P < 0.0001$) and in the intermediate age group ($P < 0.01$). The percentage of B cells did not increase until day 13 p.i. and the oldest age group showed the strongest increase. The percentage of B-cells was, as mentioned (Section 3.5.1), initially lowest in the youngest age group, however this difference disappeared, as there was an increase in percentage of B-cells in the young age group from day 4 p.i. From day 18 p.i. and onward (until end of measurements day 77 p.i.), the percentages of B cells were similar in all three age groups (data not shown).

For percentage of CD2+ lymphocytes there was a distinct increase in the intermediate age group while the other two groups showed more moderate increases. At day 4 p.i., the percentage of CD2+ cells in the intermediate age group and the youngest age group were both significantly higher ($P < 0.05$) than the mean CD2+ percentage of the oldest age group. Percentages of CD8+ cells and γδ-cells peaked days 4 and 6 p.i. respectively, but did not vary significantly according to age.

No significant differences in absolute numbers of leukocyte subset (cells per ml) (not shown) was found in any subsets apart from B cells that were still significantly lower in the youngest age group. For CD2, CD4, CD8 and B cells a drop in absolute numbers could be seen at day 6 p.i. showing the lymphopenia also shown in Fig. 4 (data not shown).

3.6. Intracellular staining of cytokines after specific stimulation

Intracellular staining for production of cytokines after BRSV stimulation of PBMCs in vitro was performed for IL-6, IL-10, TNFα and IFNγ in all animals at days −7, 11, 13, 21 and 28 p.i. However, the days −7 and 13 p.i. were chosen as representative (Fig. 6). On day −7 p.i. responses were absent or very low, however, one animal from the oldest age group had a low specific production of all cytokines on this day. On day 13 p.i., the youngest age group had the highest specific IL-6 and TNFα response.

The differences in specific TNFα were statistically significant between the youngest age group (mean 4.27 %) and the oldest age group (mean −0.84%) (slightly negative after subtraction of PBS-control and isotype control) ($P < 0.05$) and between the youngest age group (4.27%) and the intermediate age group (−0.41%) group ($P < 0.01$).

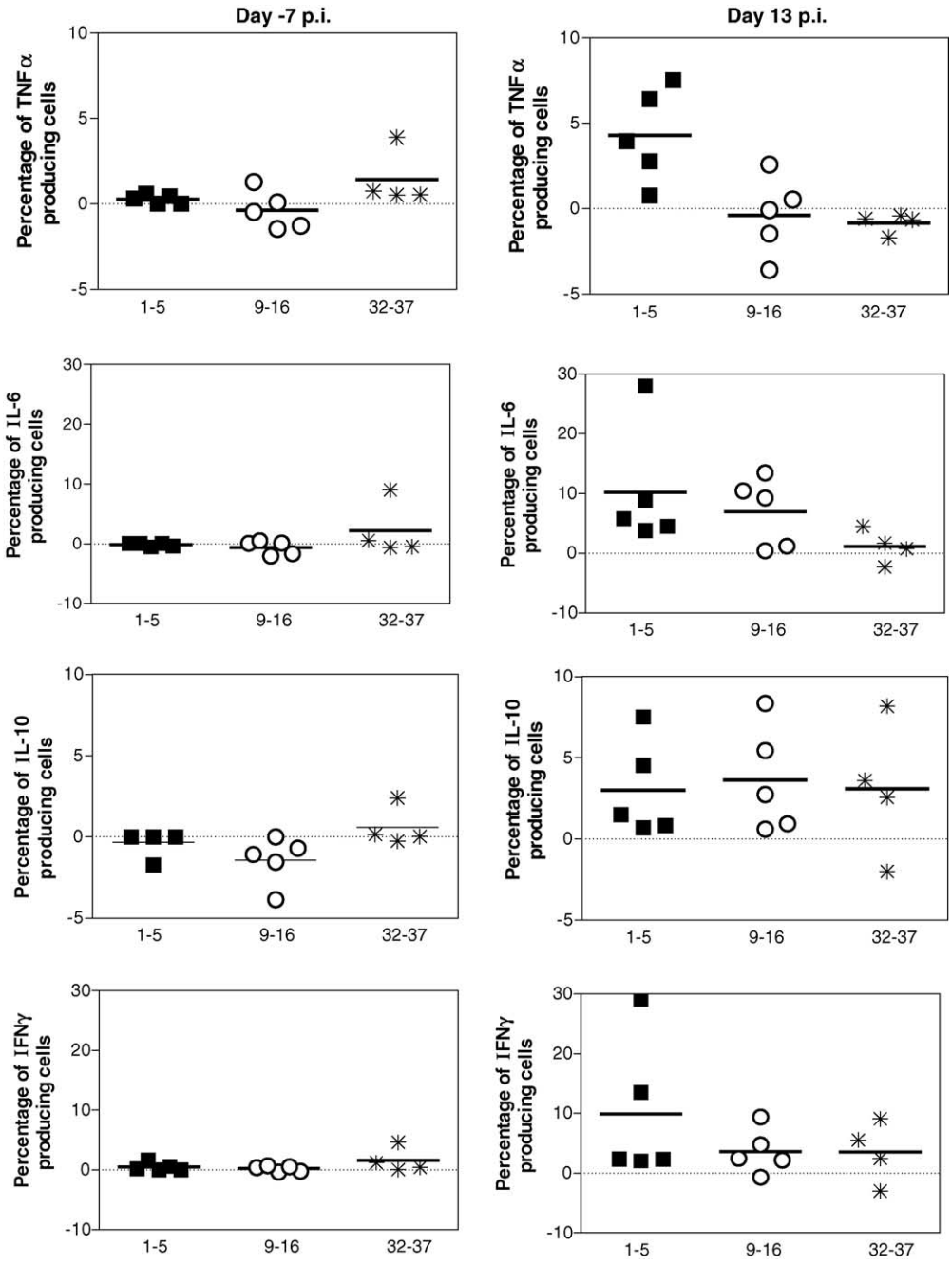

Fig. 6. Intracellular staining for TNFα, IL-6, IL-10 and IFNγ after in vitro stimulation with BRSV antigen. Results are shown for day −7 p.i. (left panel) and day 13 p.i. (right panel). *Y*-axis shows percentage of positive cytokine producing cells after in vitro stimulation with BRSV antigen subtracteing positive cytokine producing cells after in vitro stimulation with control antigen. Black squares represent the youngest age group (1–5 weeks old), circles represent the intermediate age group (9–16 weeks old) and the asterisks represent the oldest age group (32–37 weeks old).

The higher specific IL-6 responses in the youngest age group (mean 10.15%) were not statistically higher than the responses in the intermediate age group (mean 6.95%) and the oldest age group (mean 1.15%). The specific IFNγ responses were highest (non-significant) in the youngest age group (mean 9.9%) compared with the intermediate age group (3.6%) and the oldest age group (3.5%), while the specific IL-10 responses were similar in all age groups.

3.7. Specific IFNγ response measured by ELISA

Supernatants from BRSV specific antigens in vitro stimulated PBMC's (3×10^6 cells/ml) were tested in an IFNγ ELISA (Fig. 7).

Before infection, no animals showed specific IFNγ response to in vitro stimulation with BRSV antigen (Fig. 7). After infection, specific IFNγ responses were measured from

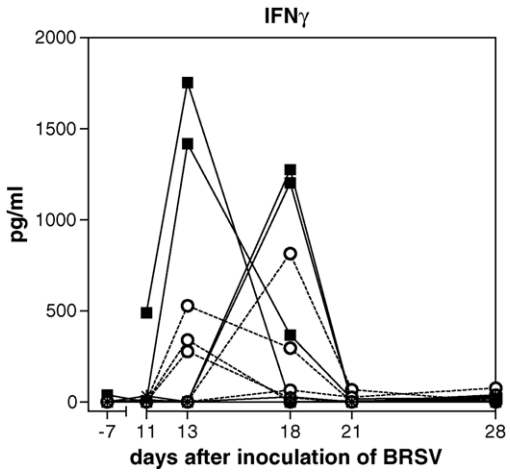

Fig. 7. Antigen-specific production of IFNγ after in vitro stimulation with BRSV antigen. Black squares represent the youngest age group (1–5 weeks old), circles represent the intermediate age group (9–16 weeks old) and the asterisks represent the oldest age group (32–37 weeks old).

day 11 p.i., where one animal from the youngest group had a specific IFNγ response. On days 13–18 p.i., increased specific IFNγ responses were detected in the young and the intermediate age groups, but not in the oldest group. At days 21 and 28 p.i. there was no response in any of the groups. Control stimulations with SEB induced IFNγ responses in all calves on all the days measured including days 21 and 28 p.i. Measuring the level of the specific IFNγ response as either peak values or as the area under the curve, a significant difference between the youngest group and the oldest group ($P < 0.01$) was found; the mean for peak values the youngest age group was 1139 pg/ml after stimulation with BRSV antigen, 405 pg/ml for the intermediate age group and 42 pg/ml for the old age group.

Measuring the specific IFNγ response after stimulation of whole blood (1.5 ml blood without regarding cell counts) a similar differentiation between age groups was found, though not as significant. In whole blood stimulation assay, two animals from the oldest group had specific IFNγ response around day 13 p.i. (data not shown).

4. Discussion

Respiratory syncytial virus is one of the major causes of viral lower respiratory tract disease in infants and calves. Age is considered a risk factor for development of severe lower respiratory tract infections by RSV in infants [30], and similarly most epidemiological studies have concluded that mainly young calves are severely affected by BRSV infection [17,18]. The results of the present study confirm this in an experimental infection model in cattle. Although not the natural host for RSV the mouse model has been very successful in reproducing many aspects of the human disease and has been used extensively to study the immunology of RSV. The importance of age has also been investigated in the mouse model, suggesting that age at first infection play a key role in shaping later immune responses [31]. However, the RSV infection in mice differs in a number of respects from that in the natural hosts as both active virus replication and virus-induced inflammation is limited [23].

In the bovine experimental infection model used here, mock inoculated animals, i.e. animals inoculated with cell cultured without virus [32,33], and non-treated animals [34] showed no signs of disease, no acute phase response and no cellular or cytokine changes, and it was therefore chosen here to use each individual animal as its own control (before infection samples).

Significant differences in clinical symptoms were found between three age groups (1–5, 9–16 and 32–37 weeks), where the youngest animals developed more severe clinical symptoms than the older calves. Some of the differences could be accounted for by differences in the balance of the immunological response between an antibody based (Th2) and a cellular response (Th1), although maternal antibodies may also play a role. Large differences in the levels of neutralizing antibodies and IgA were found between age groups (Fig. 2). The oldest age group had the highest neutralizing antibodies and IgA antibody response, both differences were significant different. Initial maternal IgG1 antibodies could be seen in the intermediate and the young age group, whereas the oldest age group had no maternal antibodies similar to what has previously been reported [35]. The protective effect of maternal antibodies has been questioned [36], and such antibodies were even found to suppress both local and systemic antibody responses [37]. Kimman et al. (1987) found that maternal IgG1 antibodies caused suppression of all isotypes, however this suppressive effect was rarely total, especially with respect to other isotypes than IgG1 [37] (own observations). Thus, it is likely that the observed differences in IgA and in neutralizing responses between the different age groups in the present study could be due to the fact that the older animals were better IgA responders. This is in line with previous reports, where it was found that older animals were capable of mounting a stronger local IgA response in the lung [3] and studies which showed that neonatal calves lacked IgA in the lung [36].

Also, human studies have showed that infants have an impaired antibody response, compared to older children in response to RSV [38–40]. Furthermore, these studies showed that the antibodies that were produced in the first half year of life favoured linear epitopes which apparently had a detrimental role, as it predisposed the infants to get ill [19,20]. In addition, several studies have shown that the weak or absent antibody response was due to lack of protective antibodies against glycoproteins. Protecting antibodies of isotype IgG2 and IgG4 against glycosylated epitopes was not acquired after infection [41,42]. In the present study, highly significant differences in number of B-cells between groups were found. The youngest age group had up to four to five times fewer B-cells than the oldest age group, which may explain some of the difference in antibody response between the age groups.

Other bovine studies comparing newborn and older calves showed the same age-dependent difference in the amount of mature immunoglobulin (IgA and IgG) producing cells in the ileal intraepithelial lymphocyte population [43] and in the tracheobronchial tree [36].

Impaired Ig production can, however also be due to a lack of help from T-cells [21]. In the present study the oldest animals had significantly higher numbers of white blood cells, especially lymphocytes than the younger animals confirming other studies [44,45]. Also in the present study, higher percentages of CD2+ and CD4+ cells in young calves compared to old calves were found. Similar results have been found in blood and lymphoid tissues of foetal calves compared to maturing calves and adult cows [22]. Also age independent fluctuation of γδ+ cells were comparable to the finding in other studies [46] however a significant rise in CD8+ and a reduction in CD4+ cells as previously reported in experimentally BRSV-infected lambs [47] could not be seen. Other factors that may influence the outcome of infection are the extent of the inflammatory response and the capacity to produce cytokines could also influence the susceptibility to BRSV infection. It can be expected that a less aggressive inflammatory response will lead to fewer clinical symptoms but also will be less efficient in clearing the infection. A very strong inflammatory response may be very efficient in killing the virus, but will also induce host pathology. Human studies have suggested that RSV infection in elderly, which are highly susceptible to RSV, are associated with a disregulation of the inflammatory response with prolonged inflammatory activity with high levels of inflammatory cytokines [48]. A similar situation could exist in infants with an immature immune response. The present study may imply that similar problems in controlling the inflammatory response occur during infection with BRSV, as the initial percentage of granulocytes in the youngest age group of calves were significantly higher than in the older age groups. In this age group inflammatory cytokines, including TNFα and IL-6 were also more dramatically induced at day 13 p.i. Also the higher fever and the higher percentage of granulocytes in the youngest age group of calves are indicative of a stronger inflammatory response. Several reports have focused on RSV and inflammation; it has previously been shown that the magnitude and duration of the acute phase protein response (haptoglobin) were related to the severity of clinical signs (fever) and with the extent of lung consolidation in experimental BRSV infection [32]. Human studies have shown that RSV is a potent inducer of inflammatory cytokines [49,50]. Other human studies have further shown that neonatal individuals can express IL-6 and TNFα in response to RSV [51]. In the bovine system, TNFα and acute phase response have both been found in BRSV infection peaking at the time with most clinical sign at day 7 after infection, which fits well with the expected involvement of these proteins in the tissue affecting part of the pathogenesis [33].

Taken together these findings imply that the magnitude of the inflammatory response has a vast impact on the clinical response, and that the proinflammatory cytokines and acute phase proteins associated with this response are probably, as reported elsewhere (Grell et al., in press), connected with and influencing the later specific response to the virus infection. Age-dependent variation in the capacity to produce inflammatory cytokines and acute phase proteins may, therefore, explain the differences between age groups.

Lastly, age-dependent variation in T-cell produced cytokines may also influence the clinical differences between age groups. It has been reported that the ability of infants to mount an IFNγ response increases between birth and the age of 5 years and that the IFNγ production is delayed until after the age of 1 year [21]. These results are in contrast to the present study where the IFNγ response after specific stimulation of PBMCs measured both by ELISA and by intracellular staining was higher in the youngest age group comprising the most severely affected calves. Whether the differences in the severity of clinical symptoms are caused directly by the differences in the IFNγ response or vice versa is not known. It should, however, be noted that the specific IFNγ response in whole blood (tested by ELISA, not shown) showed less substantial differences between age groups, probably due to the use of whole blood instead of a fixed number of PBMCs as used here.

These results on IFNγ, indicating the presence of a seemingly non-protective cellular response seem to be in conflict with several previous studies. Firstly, the general dogma that Th1 like immunity is necessary in order to achieve protective immunity towards virus infections. Secondly, that a cell-mediated cytotoxic CD8+ response is essential for clearance of the virus [4]. And thirdly, that severe RSV infection is associated with a shift in the balance from Th1 towards Th2 cytokines [52] (bovine) [53] (human). However, studies on infants have suggested that RSV-induced wheezing is characterized by an immunologic imbalance resulting in excessive release of IFNγ [40,54], and other studies have shown that IFNγ knockout mice exhibited less severe signs of airway obstruction [55]. Despite the fact that controversy exist, IFNγ seems to play a possible protective role in terms of limiting viral replication but also a pathogenic role in causing airway obstruction. The Th1/Th2 paradigm may be an oversimplification of a much more complex immunoregulatory network, in which both antibody-based and cellular-based responses are necessary for the combat of RSV. In favour of this is the observed equal production of IL-10 in all age groups, indicating as reported before, that bovine IL-10 may have an immunoregulatory effect that is independent of age [56].

Thus, while a cytotoxic response was clearly indicated as being of importance for protection against infection with BRSV by eliminating the virus [4], the findings here support that neither specific IFNγ nor proliferative responses in the blood (not shown), after in vitro recall stimulation with BRSV antigen, are key parts of the cellular immunology mediated protection against disease. As several previous observations suggest that antibodies alone do not bring about protection [5], protection must be mediated by other factors, or combinations of factors, possibly locally in the lungs.

In conclusion, in the current study the oldest animals with less clinical symptoms had a predominantly humoral immune response with the highest percentage of B cells and the highest titres of IgG and IgA, whereas the youngest animals had a predominantly inflammatory and cellular response with the highest fever and respiratory rate, the highest inflammatory specific cytokine response and the highest specific cytokine induction. It may be speculated that the initiation of an exaggerated inflammatory cytokine response by itself can induce or exacerbate the clinical symptoms of a BRSV infection. However the true cause-effect relationship remains to be investigated.

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