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Pivotal role of ChIFN γ in the pathogenesis and immunosuppression of Infectious Bursal Disease

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Pivotal role of ChIFN γ in the pathogenesis and immunosuppression of Infectious Bursal Disease

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Summary

This study investigates the pivotal role of chicken interferon-gamma (ChIFN γ) in the pathogenesis and immunosuppression of infectious bursal disease virus (IBDV) infection and is divided into *in vivo*, *ex vivo* and *in vitro* experiments. Two-week-old specific pathogen free (SPF) chickens were inoculated with the 849VB very virulent strain of IBDV (vvIBDV). The levels of systemic ChIFN γ and chicken interleukin-6 (ChIL-6) in the serum were followed for two weeks during *in vivo* experiments. Then, splenocytes and bursal cells from infected chickens were analyzed for their immunocompetence after mitogenic activation in *ex vivo* experiments. Finally, *in vitro* experiments were conducted to assess the direct immunosuppressive effect of ChIFN γ on splenocytes and peripheral blood lymphocytes (PBL) from non-inoculated SPF chickens. Our results reveal that the acute phase of infectious bursal disease (IBD) coincides, on one hand, with high levels of systemic ChIFN γ and ChIL-6 and, on the other hand, with a strong inhibition of proliferation and activation of mitogen-stimulated splenocytes from infected chickens, as measured by ChIFN γ production. Two weeks after viral inoculation, T-lymphocytes infiltrating the bursa of Fabricius (BF) had recovered their activation capability. Finally, an *in vitro* study showed that the proliferation of naïve splenocytes and PBL's was directly and specifically inhibited by ChIFN γ . As conclusion, a ChIFN γ dysregulation occurs in chickens infected with IBDV

and the overproduction of ChIFN γ by T-lymphocytes plays a key role in the pathogenesis and immunosuppression induced by this virus.

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Introduction

Infectious bursal disease virus (IBDV), a member of the *Birnaviridae* family whose genome consists of two segments of double-stranded RNA, causes an acute, highly contagious disease of young chickens, mainly at the age of 3-6 weeks (van den Berg, 2000; van den Berg *et al.*, 2000). The virus is responsible of considerable economic losses to the poultry industry world-wide by causing a high rate of morbidity and mortality in an acute form or as a consequence of severe immunosuppression. Indeed, IBDV has a predilection for the actively dividing and differentiating immature B-cells within the bursa of Fabricius. Chickens infected by IBDV develop marked depletion of B-lymphocytes bearing immunoglobulin (Ig)M on their surface which leads to severe B-cell suppression during the acute phase of IBDV infection (Nieper *et al.*, 1999; Kong *et al.*, 2004). In addition to lysis, it has been postulated that the B-lymphocyte depletion in this organ is also caused by inhibition of proliferation and induction of apoptosis (Nieper *et al.*, 1999; Rodriguez-Lecompte *et al.*, 2005). During the acute phase, the infected chickens show prostration, ruffling of feathers, dehydration and watery diarrhoea. Usually, mortality starts on the 2nd and the 3rd day after infection, reaches a peak at day 4, and then drops quickly. The surviving chickens recover rapidly with a state of apparent health after 5 days (Butter *et al.*, 2003; van den Berg *et al.*, 2004; Williams & Davison, 2005). Mortality rate is highly variable and depends, among other factors, upon the virulence and dose of the viral strain, as well as on the age and breed of the birds. In a fully susceptible flock (3 to 6-week-old chickens), the cause of clinical signs and death is still unclear and does not seem to be only related to the severity of the lesions and the bursal damage. Indeed, after infection, some birds with few bursal lesions can be found dead, while others survive despite extensive bursal damage.

Prostration, with ruffled feathers, diarrhoea and loss of appetite preceding death is reminiscent to what is observed in acute coccidiosis, where it has been shown that ChIFN γ is likely to play an important role in the onset of the clinical signs and to be involved in the different breed susceptibility to the disease (Rothwell *et al.*, 2000; Yun *et al.*, 2000). Notably, during the course of coccidiosis, an increased secretion of ChIFN γ has been demonstrated in the spleen, caecal tonsils, intestinal intraepithelial lymphocytes (Yun *et al.*, 2000) and in the gut (Rothwell *et al.*, 2000). During the acute phase of infectious bursal disease (IBD), while bursal follicles are depleted in B-cells, T-cells accumulate at and near the site of virus replication (Tanimura & Sharma, 1997; Kim *et al.*, 1998, 2000; Sharma *et al.*, 2000). The IBDV-induced bursal T-lymphocytes are activated and exhibit up-regulation of cytokines genes, such as ChIFN γ (Kim *et al.*, 2000). As a consequence, the ChIFN γ production could activate macrophages to produce inflammatory factors, such as nitric oxide (NO) and IL-6, that may promote cellular dysregulation and also accentuate tissue destruction (Digby & Lowenthal, 1995; Karaca *et al.*, 1996; Kim *et al.*, 1998). Although an increased level of IFN γ is observed during the septic shock-like syndrome in mammal and human serum (Hack *et al.*, 1997), similar observations in the chicken have, so far, never been reported at a systemic level but only locally, in the lymphoid organs, after IBDV infection (Kim *et al.*, 1998, 2000; Sharma *et al.*, 2000). Moreover, macrophages and monocytes may be infected by IBDV and then be directly activated and serve as virus carriers from the site of infection in the gut to the bursa and other peripheral tissues (Kim *et al.*, 1998; Lam, 1998; van den Berg, 2000; Khatri *et al.*, 2005; Khatri & Sharma, 2006; Palmquist *et al.*, 2006). For all these reasons, it can be postulated that ChIFN γ and macrophage pro-mediators might play a synergic role in susceptibility to IBD and immunosuppression by the direct activation of macrophages after IBDV infection and/or by their indirect activation via ChIFN γ produced by T-cells.

In addition, although the immunosuppression caused by IBDV is directed principally towards B-lymphocytes and that T-cells do not serve as primary targets for infection and replication of IBDV (Kim *et al.*, 2000), an effect on cell-mediated immunity (CMI) has also been shown (Sharma & Fredricksen, 1987; Sharma *et al.*, 1989; Cloud *et al.*, 1992a, 1992b). Immunosuppressive effect on CMI has been demonstrated *ex vivo* by using lymphocyte proliferation tests (Confer *et al.*, 1981; Confer & MacWilliams, 1982; Sharma & Lee, 1983; Karaca *et al.*, 1996; McNeilly *et al.*, 1999) but not yet by checking *ex vivo* ChIFN γ production after mitogenic activation of lymphocytes.

The present study investigates the role of ChIFN γ in the pathogenesis and immunosuppression induced by IBDV. In this aim, *in vivo*, *ex vivo* and *in vitro* experiments have been performed. Two-week-old SPF chickens were infected with a vvIBDV strain and examined during the acute phase of the disease and after recovery. The IBDV pathogenesis was studied by following mortality, morbidity, IBDV serology as well as systemic ChIFN γ and ChIL-6 levels in serum, whereas the immunocompetence of IBDV-infected chickens was analyzed after mitogenic activation of splenocytes and bursal cells. Finally, the direct role of ChIFN γ in the induction of immunosuppression of lymphocytes was evaluated by culture of naïve immunocompetent cells pre-incubated with ChIFN γ and then activated by mitogens.

Materials and methods

Chickens and virus. SPF white Leghorn chickens used in this study were hatched from eggs provided by Lohmann Valo (Cuxhaven, Germany) and kept in isolators. The 849VB

vvIBDV strain used for inoculating chickens was isolated in Belgium in 1987 and described in van den Berg *et al.* (1991, 2004).

Reagents. The mitogens phorbol 12-myristate 13-acetate (PMA), ionomycin and phytohaemagglutinin (PHA) were purchased from Sigma (Belgium). Production of *Escherichia coli*-derived recombinants via prokaryotic expression vector (ProEx) (Invitrogen Life Technologies, Belgium), rec-ChIFN γ and rec-ChIL-2, and of neutralizing mAb specific to ChIFN γ were described in detail in Lambrecht *et al.* (1999, 2004).

Isolation of splenocytes, bursal cells, PBL, and lymphocytes proliferation assay. Spleen and bursa were aseptically removed from chickens and were squeezed with a 5-ml syringe plunger to extrude cells. Cell suspensions were filtered through 70 μ m nylon (Falcon BD Biosciences, Belgium) before being washed three times in RPMI 1640 medium and resuspended in the same medium supplemented with 2 % inactivated foetal calf serum (FCSi).

Blood was taken from the wing vein in 100 U/ml of heparin (Sigma). Blood samples were incubated for 30 min at room temperature with a concentration of 0.1 g/ml of iron (Sigma). PBL were separated by decantation of iron followed by Ficoll-Paque (Pharmacia, Belgium) density separation and washed three times in RPMI 1640 medium before being resuspended in RPMI medium supplemented with 20 % FCSi. Lymphocytes proliferation assay was performed as described previously (Lambrecht *et al.*, 2004)

Fluorescence-activated cell sorting (FACS) analysis of bursal cells. Pools of bursal cells from infected birds containing 5 chickens per group were examined. After suspension in saturating buffer (PBS containing 3 % of FCSi and 0.1 % of NaN₃), 10⁶ bursal cells were

incubated with anti-chicken CD3 (Southern Biotechnology, Belgium) or anti-chicken IgM mAbs (kindly provided by B. Kaspers, Institut für Tierphysiologie, University of München, Germany) on ice at 4 °C for 30 min. Following three washes in saturating buffer, the cells were stained with sheep anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) purchased from Sigma. Subsequently, cells were washed in saturating buffer and suspended in PBS. Positively stained cells were analyzed by FACS Vantage SEM and WinMDI 2.8 software. Viable bursal cells were gated on the basis of forward and side scatter characteristics, and 10 000 events were analyzed for positive staining with FITC.

ChIFN γ ELISA assay. The capture ChIFN γ specific ELISA was described previously (Lambrecht *et al.*, 2000, 2004) and now commercially available from Biosource Europe (Belgium, catalogue number #CAC1233). It was performed on undiluted supernatant of mitogenic stimulated cells or sera from infected or non-infected birds (diluted at 1:100), following the manufacturer's instructions. According to the standard curve provided in the kit, an optical density (O.D.) of 1 corresponds to 1.33 ng/ml of ChIFN γ .

ChIL-6 bioassay. ChIL-6 level in serum was measured by using the IL-6-dependent murine hybridoma 7TD1 cell line (kindly provided by J. Van Snick, Ludwig Institute for Cancer Research, Brussels, Belgium). The ChIL-6 bioassay was performed as described previously by Schneider *et al.* (2001) with several modifications. Briefly, 7TD1 cells were harvested and washed three times in warm PBS. Cells were adjusted to 10⁵ cells per well and serial two-fold dilutions of serum samples starting at 1:100 and of recombinant ChIL-6 (Schneider *et al.*, 2001) starting at 10 ng/ml as standard were added in triplicate. After 72 h of incubation at 37 °C, 100 μ l of cell supernatant were removed from each well and replaced with 10 μ l/well of tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-

tretrazolic]1,3-benzene disulfonate) (Roche, Belgium). After incubation for 4 h, O.D₄₅₀ was determined with an ELISA reader. Proliferation responses were calculated as the mean of triplicate wells.

Measurement of the IBDV humoral response by ELISA. The IBDV specific ELISA test was performed essentially as previously described (van den Berg *et al.*, 2001) with several modifications. In short, microtiter plates were coated with vp2-IBDV specific mAb 1B2 at 1 µg/ml in PBS for 1 h at 37 °C. After blocking, plates were then incubated with vp2-IBDV protein (849VB vvIBDV strain) produced in baculovirus system. Serial two-fold dilutions of samples of serum starting at 1:100 were added. The chicken IgG specific rabbit antibody coupled to horseradish peroxidase (Sigma) was utilized as the detection reagent.

Experimental design. *In vivo* experiments. Sixty three 2-week-old SPF chickens were inoculated with 849VB vvIBDV strain whereas 40 chickens received only PBS. They were identified as IBDV-infected and negative birds, respectively. Chickens received 10³ median embryo infective doses (EID₅₀) of vvIBDV strain via the oculo/nasal route. In previous study, this challenge dose did not induce more than 50 % mortality in fully susceptible young birds (2-weeks-old). Surviving birds were sacrificed and blood sampled at 2, 3, 4, 5, 9 and 16 days post-infection (pi). For each harvested serum, IBDV serology, circulating ChIFN γ and ChIL6 levels were measured by specific ELISAs and bioassay described in previous sections. The criteria used to consider a bird as sick were prostration, dehydration, watery diarrhoea and/or ruffled feathers.

***Ex vivo* experiments.** In parallel to the *in vivo* experiments, spleen and bursa of Fabricius were taken at days 4, 9 and 16 pi from each IBDV-infected and negative chicken used in the *in vivo* experiment. Splenocytes and bursal cells were isolated for *ex vivo* mitogen-

stimulation and immunosuppression evaluation. Lymphocytes were either grown in culture medium alone or stimulated with different T-cell mitogens at optimal concentrations as previously described in Lambrecht *et al.* (2004) : a blend of PMA/Ionomycin (0.1/0.1 $\mu\text{g/ml}$), PHA (10 $\mu\text{g/ml}$) or rec-ChIL-2 (10 $\mu\text{g/ml}$). All tests were performed in triplicate. T-cell stimulation was measured by lymphocyte proliferation and ChIFN γ production after 48 h of incubation at 39°C. In addition, at days 4 and 16 pi, the phenotype of immune bursal cells was determined by specific staining of B- and T-cells and FACS analysis.

In addition, two further *ex vivo* experiments were conducted to confirm the results of the first trial (immunosuppression of splenocytes as measured by the absence of proliferation and ChIFN γ production after mitogenic activation). In each of these experiments, 20 chickens were inoculated with 849VB vvIBDV strain whereas 10 and 11 chickens, respectively, were used as negative controls. Birds were sacrificed and spleens were taken at days 4 and 16 pi. Splenocytes were isolated and activated with PMA/Ionomycin or ChIL2 mitogens as previously described.

In vitro experiments. Spleen and blood were taken from four 4-week-old SPF chickens for isolation of splenocytes and PBL, respectively, as previously described. Cells (10^7 cell/ml) were grown in culture medium either alone or in presence of recombinant rec-ChIFN γ (0.6 $\mu\text{g/ml}$) pre-incubated or not during 4 h with neutralizing mAb to ChIFN γ (1E12). After an overnight incubation at 39 °C, a blend of PMA/Ionomycin (0.1/0.1 and 1/1 $\mu\text{g/ml}$ ratio for splenocytes and PBL activation, respectively) or culture medium alone was added to each cell culture for a final incubation of 48 h. The *in vitro* effects of ChIFN γ on immunocompetent cells were measured by lymphocyte proliferation.

Statistical analysis. Statistical analysis of lymphocyte proliferation data was performed using the one-way analysis of variance (ANOVA) on \log_{10} -transformed count per minute (cpm) (Minitab 13 software, statistical programme for Windows, 2000) in order to analyze the difference between the groups. For the *ex vivo* ChIFN γ production by lymphocytes after mitogenic activation, ChIFN γ and ChIL-6 levels in sera and IBDV serology, a \log_{10} transformation was also performed on O.D. data before statistical analysis. Differences were considered as significant at $P < 0.05$. Coefficient of determination (R^2) was also calculated to determine if any significant relationships existed between cellular proliferation, *ex vivo* ChIFN γ production and systemic ChIFN γ and ChIL-6 in serum.

Results

In vivo experiments. *Morbidity and mortality.* After inoculation of 2-week-old SPF chickens with the 849VB vvIBDV strain, clinical signs started on the 3rd day and persisted during 3 days ([Table 1](#)). Interestingly, at days 3 and 4 pi, a clear distinction could be made between several apparently healthy (showing no symptoms) and sick birds, with high variability among severity of clinical symptoms, until day 5 pi. So, about ten per cent of IBDV-infected chickens died between the 3rd and the 5th day pi but it must be mentioned that severely sick chickens sampled at days 3 and 4 pi would probably have died if they were not sacrificed for *in vivo* and *ex vivo* experiments. Therefore, chickens showing clinical symptoms were recorded separately from healthy chicken in Table 1. The convalescent birds recovered rapidly to an apparent state of health. A humoral response was detected 9 days pi by vp2-IBDV specific IgG ELISA ([Table 1](#)).

Systemic cytokines. Sera from IBDV-infected and negative birds were also taken in order to monitor systemic ChIFN γ and ChIL-6 levels ([Table 1](#)). Significant levels of ChIFN γ were detected in the serum of IBDV-infected chickens during a few days pi, corresponding to the onset of the clinical symptoms in inoculated group ($P < 0.05$). It was noteworthy, however, that the ChIFN γ levels increased at day 2, one day before the appearance of the first clinical symptoms, and that the higher ChIFN γ levels was detected in serum of sick IBDV-infected chickens at day 3 pi ($P < 0.05$).

The highest levels of systemic ChIL-6 were also observed in inoculated birds a few days pi ([Table 1](#)), although the ChIL-6 levels increased before the appearance of the first clinical symptoms and that the difference between negative and IBDV-infected chickens was only significant at days 3 and 5 pi ($P < 0.05$). However in contrast to measured ChIFN γ levels, higher background of ChIL-6 levels were observed in the serum of negative chickens during the whole experiment, making some interpretations more difficult.

Ex vivo experiments. *Immunosuppression of splenocytes.* PMA/Iono in combination, PHA and ChIL-2 were used to activate splenocytes from IBDV-infected chickens at 4, 9 and 16 days pi, as measured by *ex vivo* proliferation and ChIFN γ production ([Table 2](#)). At the 4th day pi, splenocytes from IBDV-infected chickens neither proliferate nor produce ChIFN γ after *ex vivo* mitogenic activation ($P < 0.05$). At day 9, a regeneration of the activation ability of splenocytes was observed, however, some IBDV-infected birds still showed immunosuppression, as revealed by high standard deviation values at the 9th day pi. It is interesting to note that splenocytes from neither negative nor IBDV-inoculated chickens did exhibit any *ex vivo* spontaneous proliferation and ChIFN γ production, as shown by the results of non-activated splenocytes in [Table 2](#).

Splenocytes harvested from infected chickens showing high level of systemic ChIFN γ in serum produced no ChIFN γ after *ex vivo* mitogenic activation ($R^2 = 0.49$ with PMA/Iono activation), On the contrary, IBDV-infected birds with highest levels of systemic ChIL-6 did not show the most important evidence of immunosuppression (individual data not shown).

Immunosuppression of bursal cells. In a preliminary experiment, different mitogens and concentrations were tested for their ability to activate bursal cells from SPF chickens. A blend of PMA and Ionomycin at 0.1/0.1 $\mu\text{g/ml}$ ratio appeared to be the optimal mitogenic activation for the highest proliferation (data not shown). This mitogenic combination was used to activate bursal cells of negative and IBDV-infected chickens at days 4, 9 and 16 pi. In the IBDV-infected group, only a weak proliferative response was restored at 16 days pi ([Figure 1](#)) and surprisingly, at day 16 pi, ChIFN γ was detected in supernatant of these mitogen-activated bursal cells. As expected, a high proliferation of mitogen-activated bursal cells was observed in the negative group during the whole experiment and proliferating bursal cells were not able to produce ChIFN γ after *ex vivo* mitogen-activation ([Figure 2](#)). The individual data of IBDV-infected chickens at day 16 showed that the bursal cells cultures with the highest proliferation response correlated with the highest level of ChIFN γ production and *vice versa*, as confirmed by an important correlation ($R^2 = 0.81$).

As shown in FACS analysis of bursal cell population, the proportion of B-cells in the bursa of uninfected chickens ranged from 78 to 82 % of the total bursal cell population (data not shown). In IBDV-infected group, the level of B-cells remained at this range of total bursal cells 4 days pi (data not shown) but in contrast, at day 16 pi, the proportion of bursal B-cells dropped to 15 % (data not shown). The bursal T-cells numbers were analysed at day 16 pi in IBDV-infected chickens. We observed that T-cells reached 15 % of the total bursal

cell population in IBDV-infected chickens ([Figure 3B](#)) whereas this proportion constituted less than 2 % in negative birds ([Figure 3A](#)).

***In vitro* experiments.** The role of ChIFN γ in immunosuppression was evaluated by incubation of recombinant ChIFN γ (rec-ChIFN γ) in culture medium of *ex vivo* mitogenic activation of splenocytes and PBL from immunocompetent 4-week-old SPF chickens ([Table 3](#)). Interestingly, a significant reduction of splenocyte and PBL proliferation was observed when cells were incubated with rec-ChIFN γ ($P < 0.05$). This immunosuppressive activity was abrogated when a neutralizing mAb specific to ChIFN γ was supplemented to the rec-ChIFN γ . The specificity of these observations was confirmed by the absence of immunosuppression induced by an irrelevant protein expressed using the same prokaryotic system, and also by the observation of immunosuppression when rec-ChIFN γ activity was not neutralised by the use of non-specific mAb, on the other hand.

Discussion

It is well known that IBDV has an immunosuppressive effect on the humoral immunity by destructing premature B cells in the BF. More recently, several studies have demonstrated its impact on the cell-mediated immunity (Sharma *et al.*, 2000). In this context, using a capture ELISA specific for ChIFN γ , our paper is the first study showing the appearance of high ChIFN γ levels in the blood a few days after vvIBDV infection and the following subsequent T-cell unresponsiveness.

A first set of *in vivo* experiments showed the highest levels of ChIFN γ and ChIL-6 in the blood of IBDV-infected chickens coinciding with the acute phase of IBD. These cytokines are indicative of the T-cell infiltration of the lymphoid organs and the exacerbated inflammatory response that occurs locally, at the site of viral multiplication (Tanimura & Sharma, 1997; Kim *et al.*, 1998, 2000; Sharma *et al.*, 2000). In this way, ChIFN γ most probably contributes to the activation of the macrophages, as shown by Kathri *et al.* (2005, 2006), which will then produce pro-inflammatory mediators (such as ChIL-6, TNF α ,...) inducing a septic shock-like syndrome in chickens during the acute phase of IBD. This cytokine storm is followed by the recovery of the birds to a state of apparent health (Butter *et al.*, 2003; van den Berg *et al.*, 2004; Williams & Davison, 2005). As mock-infected chickens remained healthy after injection of high concentration of recombinant ChIFN γ (Rauw *et al.*, 2007), it would suggest that this cytokine is more an indicator of the intense local pathological process during the acute phase of IBD than the direct cause.

Secondly, in the spleen, a set of *ex vivo* experiments confirmed previous observations showing the absence of mitogenic proliferation of splenocytes from IBDV-infected chickens 4 days after IBDV infection (Confer *et al.*, 1981; Confer & MacWilliams, 1982; Sharma & Lee, 1983; Karaca *et al.*, 1996; McNeilly *et al.*, 1999), but revealed, for the first time, the inability of these splenocytes to produce ChIFN γ at this time point, whichever T-mitogen was used for *ex vivo* activation. Their immunocompetence was already restored in some birds at day 9 pi but was completely recovered in all birds only at day 16 pi, indicating individual susceptibility. Moreover, the absence of spontaneous *ex vivo* production of ChIFN γ (without mitogen activation) by splenocytes at and after 4 days pi confirms that the overproduction of ChIFN γ took place before. Similar observations were done in the bursa of Fabricius where, contrary to control SPF chickens where B-cells constitute the major population of lymphocytes and are able to proliferate but not to produce ChIFN γ after *ex*

in vivo mitogenic activation, infiltrated T cells constitute the majority of the bursal population after IBDV infection (Kim *et al.*, 1998, 2000; Sharma *et al.*, 2000). At days 4 and 9 pi, neither proliferation -as previously shown by McNeilly *et al.* (1999)- nor ChIFN γ production were observed in infected birds, confirming the unresponsiveness of T-lymphocytes infiltrating the BF at day 4 pi combined with the B-cell depletion. Interestingly, in agreement with the results observed in the spleen, the proliferation of the bursal cells from IBDV-infected chickens was restored at day 16 pi, but conversely to virus-free birds, ChIFN γ production was also detected.

Thirdly, *in vitro* experiments demonstrated that rec-ChIFN γ was able to directly inhibit the mitogenic proliferation of spleen and blood cells from naïve chickens. This inhibitory effect cannot be attributed to macrophages and their NO production, contrary to what has been suggested in mouse (Albina *et al.*, 1991) and rat (Fecho *et al.*, 1994; Mills, 1994), because only few macrophages are detected in suspension of chickens spleen and blood cells by FACS analysis (data not shown), as these cells, unlike lymphocytes, remain strongly attached to the splenic reticular network (Mast, 1998) and were removed by iron decantation. Another possible explanation for the inhibition of cellular proliferation induced by ChIFN γ relies on its role on ChIL-2 production by T-cells. Indeed, in addition of being hyporesponsive to mitogens, splenocytes from IBDV-infected chickens were also shown to be deficient in secretion of ChIL-2, (Sharma & Fredricksen, 1987; Kim *et al.*, 1998). Moreover, in mice, it has been shown that IFN γ is responsible for decrease of IL-2 production by spleen lymphocytes (Gajewski & Fitch, 1988; Bradley *et al.*, 1996). This is confirmed by preliminary RT-PCR results showing a decreased and delayed ChIL-2 RNA production by naïve splenocytes and PBL treated with rec-ChIFN γ (data not shown).

In conclusion, the present study documents the ChIFN γ dysregulation occurring in chickens after IBDV infection. Very soon after IBDV infection, activated T-lymphocytes will overproduce ChIFN γ that, in turn, will stimulate macrophages for the production of pro-mediators like ChIL-6. This exacerbated cytokine response has a bearing on the symptoms and outcome of IBDV infection. Afterwards, this upregulation in T-cell and macrophage activation will be followed by a downregulation in convalescent chickens, leading to cellular immunosuppression of which duration and importance will depend on the strain and dose of virus, as well as on the age and breed of chickens. From all these observations, it can thus reasonably be concluded that overproduction of ChIFN γ plays a pivotal role in the pathogenesis and the T-immunosuppression induced by IBDV. The ChIFN γ capture ELISA might thus represent a powerful tool to evaluate the immunocompetence of the chicken in the future.

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References

- Albina, J.E., Abate, J.A. & Henry, W.L. (1991). Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation. Role of IFN-gamma in the induction of the nitric oxide pathway. *Journal of Immunology*, *147*, 144-148.
- Bradley, L., Dalton, D.K. & Croft, M. (1996). A direct role of IFN γ in regulation of Th1 cell development. *Journal of Immunology*, *157*, 1350-1358.
- Butter, C., Sturman, T.D.M., Baaten, B.J.G. & Davison, T.F. (2003). Protection from infectious bursal disease virus (IBDV)-induced immunosuppression by immunization with a fowlpox recombinant containing IBDV-VP2. *Avian Pathology*, *32*, 597-604.
- Cloud, S.S., Lillehoj, H.S. & Rosenberger, J.K. (1992a). Immune dysfunction following infection with chicken anemia virus and infectious bursal disease virus. I. Kinetic alterations of avian lymphocytes subpopulations. *Veterinary Immunology and Immunopathology*, *34*, 337-352.
- Cloud, S.S., Rosenberger, J.K. & Lillehoj, H.S. (1992b). Immune dysfunction following infection with chicken anemia virus and infectious bursal disease virus. II. Alteration of in vitro immune response. *Veterinary Immunology and Immunopathology*, *34*, 353-366.
- Confer, A.W. & MacWilliams, P.S. (1982). Correlation of hematological changes and serum and monocyte inhibition with the early suppression of phytohemagglutinin stimulation of lymphocytes in experimental infectious bursal disease. *Canadian Journal of Comparative Medicine*, *46*, 169-175.
- Confer, A.W., Springer, W.T., Shane, S.M. & Donovan, J.F. (1981). Sequential mitogen stimulation of peripheral blood lymphocytes from chickens inoculated with infectious bursal disease virus. *American Journal of Veterinary Research*, *42*, 2109-2113.

- Digby, M.R. & Lowenthal, J.W. (1995). Cloning and expression of the chicken interferon-gamma gene. *Journal of Interferon and Cytokine Research*, *15*, 939-945.
- Fecho, K., Maslonek, K.A., Coussons-Reaad, M.E., Dykstra, L.A. & Lysle, D.T. (1994). Macrophage-derived nitric oxide is involved in the depressed concanavalin A responsiveness of splenic lymphocytes from rats administered morphine in vivo. *Journal of Immunology*, *152*, 5845-5852.
- Gajewsky, T.F. & Fitch, F.W. (1988). Anti-proliferative effect of IFN γ in immune regulation. I. IFN γ Inhibit the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *Journal of Immunology*, *140*, 4245-4252.
- Hack, C.E., Aarden, L.A. & Thijs, L.G. (1997). Role of cytokines in sepsis. *Advances in Immunology*, *66*, 101-195.
- Karaca, K., Kim, I.J., Reddy, S.K. & Sharma, J.M. (1996). Nitric oxide inducing factor as a measure of antigen and mitogen-specific T cell responses in chickens. *Journal of Immunological Methods*, *10*, 97-103.
- Khatri, M., Palmquist, J.M., Cha, R.M. & Sharma, J.M. (2005). Infection and activation of bursal macrophages by virulent infectious bursal disease virus. *Virus Research*, *113*, 44-50.
- Khatri, M. & Sharma, J.M. (2006). Infectious bursal disease virus infection induces macrophage activation via p38 MAPK and NF- κ B pathways. *Virus Research*, *118*, 70-77.
- Kim, I.J., Karaca, K., Pertile, T.L., Erickson, S.A. & Sharma, J.M. (1998). Enhanced expression of cytokine genes in spleen macrophages during acute infection with infectious bursal disease virus in chickens. *Veterinary Immunology and Immunopathology*, *61*, 331-341.

- Kim, I., You, S.K., Kim, H., Yeh, H. & Sharma, J.M. (2000). Characteristics of bursal T lymphocytes induced by infectious bursal disease virus. *Journal of Virology*, 74, 8884-8892.
- Kong, L.L., Omar, A.R., Hair-Bejo, M., Aini, I. & Seow, H.F. (2004). Comparative analysis of viral RNA and apoptotic cells in bursae following infection with infectious bursal disease virus. *Comparative Immunology, Microbiology and Infectious Disease*, 27, 433-443.
- Lam, K.M. (1998). Alteration of chicken heterophil and macrophage functions by the infectious bursal disease virus. *Microbial Pathogenesis*, 25, 147-155.
- Lambrecht, B., Gonze, M., Morales, D., Meulemans, G. & van den Berg, T. (1999). Comparison of biological activities of natural and recombinant chicken interferon-gamma. *Veterinary Immunology and Immunopathology*, 70, 257-267.
- Lambrecht, B., Gonze, M., Meulemans, G. & van den Berg, T. (2000). Production of antibodies against chicken interferon- γ : demonstration of neutralizing activity and development of quantitative ELISA. *Veterinary Immunology and Immunopathology*, 74, 137-144.
- Lambrecht, B., Gonze, M., Meulemans, G. & van den Berg, T. (2004). Assessment of the cell-mediated immune response in chickens by detection of chicken interferon- γ in response to mitogen and recall Newcastle disease viral antigen stimulation. *Avian Pathology*, 33, 343-350.
- Mast, J. (1998). Organisation of the lymphoid structures of the chicken and the ontogeny of this immunocompetence. *Doctoraal proefschrift nr 378 aan de Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen van de Katholieke Universiteit Leuven*, 116-127, Leuven, Belgium.

- McNeilly, F., Walker, I., Allan, G.M. & Adair, B. (1999). Bursal lymphocyte proliferation in the presence of phorbol myristate acetate : effect of IBDV strains on the proliferation response. *Avian Pathology*, 28, 301-303.
- Mills, C.D. (1994). Molecular basis of suppressor macrophages. Arginine metabolism via the nitric oxide synthetase pathway. *Journal of Immunology*, 146, 2719-2723.
- Nieper, H., Teifke, J.P., Jungmann, A., Löhr, C.V. & Müller, H. (1999). Infected and apoptotic cells in the IBDV-infected bursa of Fabricius, studied by double-labelling techniques. *Avian Pathology*, 28, 279-285.
- Palmquist, J.M., Khatri, M., Cha, R.M., Goddeeris, B.M., Walcheck, B. and Sharma, J.M. (2006). In vivo activation of chicken macrophages by infectious bursal disease virus. *Viral Immunology*, 19, 305-315.
- Rauw, F., Lambrecht, B., François, A., Langlois, P. & van den Berg, T. (2007). Kinetic and biological properties of recombinant ChIFN γ expressed via CELV-virus vector. *Journal of Interferon and Cytokine Research*, 27, 111-118.
- Rothwell, L., Muir, W. & Kaiser, P. (2000). Interferon-g is expressed in both gut and spleen during *Eimeria tenella* infection. *Avian Pathology*, 29, 333-342.
- Schneider, K., Klaas, R., Kaspers, B. & Staeheli, P. (2001). Chicken interleukin-6 : cDNA structure and biological properties. *European Journal of Immunology*, 268, 4200-4206.
- Sharma, J.M. & Lee, L. (1983). Effects of infectious bursal disease virus on natural killer cell activity and mitogenic response of chicken lymphoid cells : Role of adherent cells in cellular immune suppression. *Infection and Immunity*, 42, 747-754.
- Sharma, J.M. & Fredricksen, T.L. (1987). Mechanisms of T cell immunosuppression by infectious bursal disease virus of chickens. *Proceedings of Avian Immunology*, 238, 283-294.

- Sharma, J.M., Dohms, J.E. & Metz, A.L. (1989). Comparative pathogenesis of serotype 1 and variant serotype 1 isolates of infectious bursal disease and their effect on humoral and cellular immune competence of SPF chickens. *Avian Diseases*, 33, 112-124.
- Sharma, J.M., Kim, I.J., Rautenschlein, S. & Yeh, H.Y. (2000). Infectious bursal disease virus of chickens : pathogenesis and immunosuppression. *Development of Comparative Immunology*, 24, 223-235.
- Tanimura, N. & Sharma, J.M. (1997). Appearance of T cells in the bursa of Fabricius and caecal tonsils during acute phase of infectious bursal disease virus infection in chickens. *Avian Diseases*, 41, 638-645.
- van den Berg, T., Gonze, M. & Meulemans, G. (1991). Acute infectious bursal disease in poultry: isolation and characterization of a highly virulent strain. *Avian Pathology*, 20, 133-143.
- van den Berg, T. (2000). Acute infectious bursal disease in poultry: a review. *Avian Pathology*, 29, 175-194.
- van den Berg, T., Etteradossi, N., Toquin, D. & Meulemans G. (2000). Infectious bursal disease (Gumboro disease): review. *Revue Scientifique et Technique – Office International des Epizooties*, 19, 527-543.
- van den Berg, T. Morales, D., Lambrecht, B. & Meulemans, G. (2001). Use of baculo-derived vp2 sub-unit protein for diagnosis and control of IBDV. In F. Coudert, R. Mulder and T. van den Berg (Eds). *Proceedings 1999 of the COST Action 839 : Immunosuppressive viral disease in poultry* (pp 89-96). Brussels, Belgium.
- van den Berg, T., Morales, D., Etteradossi, N., Rivallan, G., Toquin, D., Raue, R., Zierenberg, K., Zhang, M.F., Zhu, Y.P., Wang, C.Q., Zheng, H.J., Wang, X., Chan, G.C., Lim, B.L. & Müller, H. (2004). Assessment of genetic, antigenic and pathotypic criteria for the characterization of IBDV strains. *Avian Pathology*, 33, 470-476.

- Williams, A.E. & Davison, T.F. (2005). Enhanced Immunopathology induced by very virulent infectious bursal disease virus. *Avian Pathology*, 34, 4-14.
- Yeh, H., Winslow, B.J., Junker, D.E. & Sharma, J.M. (1999). In vitro effects of recombinant chicken interferon-g on immune cells. *Journal of Interferon cytokine Research*, 19, 689-693.
- Yun, C.H., Lillehoj, H.S. & Choi, K.D. (2000). Eimeria tenella infection induces local gamma interferon production and intestinal lymphocyte subpopulation changes. *Infection and immunity*, 68, 1282-1288.

Figure legends

Figure 1. Proliferation of bursal cells from negative and 849VB vvIBDV-infected chickens after ex vivo mitogenic activation. Bursal cells were stimulated with a combination of PMA and Ionomycin (0.1/0.1 $\mu\text{g/ml}$) and their proliferation were measured after 48 h of activation. Data represent mean \pm standard deviation of cpm. Means \pm standard deviations with superscript (*) differ significantly from the negative value ($P < 0,05$).
cpm = count per minute.

Figure 2. ChIFN γ production of bursal cells from negative and 849VB vvIBDV-infected chickens after ex vivo mitogenic activation. Bursal cells were stimulated with a combination of PMA and Ionomycin (0.1/0.1 $\mu\text{g/ml}$) and supernatants of stimulated cells were harvested after 48 h of activation. ChIFN γ production was determined by the ChIFN γ capture ELISA. Data represent mean \pm standard deviation of absorbance values. Means \pm standard deviations with superscript (*) differ significantly from the negative value ($P < 0,05$).
O.D. = Optical Density

Figure 3. Identification of bursal T-cells by FACS analysis from negative (A) and vvIBDV-infected chickens (B) at day 16 pi. Bursal cells were indirectly stained by anti-chicken CD3 mAb followed by sheep anti-mouse IgG conjugated with FITC.

Table 1 : Serology, ChIFN γ and ChIL6 levels in serum

Group	Days post-inoculation	Clinical signs	Number of chickens	ChIFN γ^a	ChIL6 ^b	vp2-IBDV specific IgG ^c
Negative	2	No	6	0.038 \pm 0.010 ^A	0.653 \pm 0.569 ^A	0.101 \pm 0.021 ^A
	3	No	6	0.031 \pm 0.008 ^A	0.993 \pm 0.909 ^A	0.125 \pm 0.014 ^A
	4	No	6	0.035 \pm 0.006 ^A	1.131 \pm 0.655 ^A	0.137 \pm 0.023 ^A
	5	No	6	0.032 \pm 0.008 ^A	1.122 \pm 0.417 ^A	0.161 \pm 0.028 ^A
	9	No	5	0.030 \pm 0.004 ^A	0.655 \pm 0.246 ^A	0.171 \pm 0.036 ^A
	16	No	5	0.040 \pm 0.009 ^A	0.771 \pm 0.400 ^A	0.131 \pm 0.009 ^A
849VB vvIBDV-inoculated	2	No	8	0.718 \pm 0.560 ^B	1.673 \pm 1.131 ^A	0.122 \pm 0.020 ^A
	3	No	8	0.427 \pm 0.283 ^B	1.703 \pm 0.821 ^A	0.137 \pm 0.040 ^A
		Yes	8	0.766 \pm 0.337 ^C	2.267 \pm 0.660 ^B	0.105 \pm 0.028 ^A
	4	No	8	0.550 \pm 0.408 ^B	1.487 \pm 1.046 ^A	0.117 \pm 0.061 ^A
		Yes	8	0.568 \pm 0.216 ^B	1.440 \pm 0.301 ^A	0.136 \pm 0.040 ^A
	5	Yes	7	0.773 \pm 0.634 ^B	2.232 \pm 0.957 ^B	0.125 \pm 0.028 ^A
9	No	5	0.058 \pm 0.013 ^A	1.455 \pm 0.863 ^A	2.770 \pm 0.248 ^B	
16	No	5	0.048 \pm 0.012 ^A	0.382 \pm 0.090 ^A	3.200 \pm 0.161 ^B	

^aData represent mean \pm standard deviation of absorbance values determined by ChIFN γ specific capture ELISA. Serum samples were diluted 1:100.

^bData represent mean \pm standard deviation of absorbance values determined by bioassay on 7TD1 cell line. Serum samples were diluted 1:100.

^cData represent mean \pm standard deviation of absorbance values determined by vp2-IBDV specific IgG ELISA. Serum samples were diluted 1:100.

Means \pm standard deviations with no common superscript differ significantly ($P < 0.05$).

Table 2. Proliferation and ChIFN γ production of splenocytes after ex vivo mitogen activation

Exp.	Mitogens	Groups	Days post-inoculation	Number of chickens	Proliferation ^a	ChIFN γ production ^b
I	Non-activated	Negative	4	6	2 210 \pm 592	0.131 \pm 0.140
			9	5	2 534 \pm 2 222	0.203 \pm .0287
		vvIBDV inoculated	16	5	3 255 \pm 1 592	0.283 \pm .0215
			4	16	760 \pm 378	0.058 \pm 0.019
			9	5	1 132 \pm 579	0.105 \pm 0.061
			16	5	1 769 \pm 606	0.063 \pm 0.016
	PMA/Iono	Negative	4	6	43 442 \pm 15 284	0.766 \pm 0.351
			9	5	97 915 \pm 30 343	2.618 \pm 0.161
		vvIBDV inoculated	16	5	86 223 \pm 7 729	2.550 \pm 0.287
			4	16	535 \pm 198*	0.060 \pm 0.020*
			9	5	43 129 \pm 42 737	2.640 \pm 0.089
			16	5	99 910 \pm 19 882	2.748 \pm 0.040
	PHA	Negative	4	6	112 062 \pm 16 309	2.206 \pm 0.666
			9	5	45 740 \pm 35 045	1.976 \pm 0.532
		vvIBDV inoculated	16	5	140 764 \pm 37 823	2.085 \pm 1.019
			4	16	508 \pm 211*	0.269 \pm 0.550*
			9	5	44 595 \pm 51 454	1.027 \pm 0.918
			16	5	12 7060 \pm 11 293	2.676 \pm 0.143
	ChIL-2	Negative	4	6	9 955 \pm 5 560	2.205 \pm 0.617
			9	5	7 331 \pm 4 638	1.908 \pm 1.039
		vvIBDV inoculated	16	5	20 016 \pm 9 193	2.298 \pm 0.729
			4	16	547 \pm 270*	0.135 \pm 0.178*
			9	5	5 661 \pm 5 274	0.834 \pm 0.994
			16	5	16 383 \pm 2 600	2.126 \pm 0.489
II	Non-activated	Negative	4	5	690 \pm 252	0.031 \pm 0.006
			16	5	2 964 \pm 1 616	0.070 \pm 0.064
		vvIBDV inoculated	4	11	340 \pm 141	0.081 \pm 0.103
			16	5	1 432 \pm 423	0.037 \pm 0.008
	PMA/Iono	Negative	4	5	57 551 \pm 24 733	2.455 \pm 0.066
			16	5	32 345 \pm 10 849	2.440 \pm 0.040
		vvIBDV inoculated	4	11	321 \pm 150	0.408 \pm 0.330*
			16	5	35 367 \pm 11 072	2.496 \pm 0.051
	ChIL-2	Negative	4	5	6 171 \pm 1 600*	1.994 \pm 0.361
			16	5	8 288 \pm 2 205	1.971 \pm 0.627
		vvIBDV inoculated	4	11	402 \pm 129*	0.266 \pm 0.361*
			16	5	5 836 \pm 2 024	0.845 \pm 0.857
III	Non-activated	Negative	4	6	328 \pm 81	0.024 \pm 0.006
			16	5	1 298 \pm 142	0.064 \pm 0.064
		vvIBDV inoculated	4	9	188 \pm 38	0.070 \pm 0.032
			16	5	2 970 \pm 1576	0.100 \pm 0.025
	PMA/Iono	Negative	4	6	37 956 \pm 14 069	2.857 \pm 0.071
			16	5	58 519 \pm 2 358	2.319 \pm 0.008
		vvIBDV inoculated	4	9	218 \pm 83*	0.385 \pm 0.418*
			16	5	62 038 \pm 34 626	2.343 \pm 0.206
	ChIL-2	Negative	4	6	2 994 \pm 1 407	0.234 \pm 0.369
			16	5	7 166 \pm 1 237	0.579 \pm 0.631
		vvIBDV	4	9	279 \pm 159*	0.085 \pm 0.059*

inoculated	16	5	$14\,281 \pm 10\,903$	2.159 ± 0.277
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^aData represent mean \pm standard deviation of count per minute values determined by proliferation of splenocytes after *ex vivo* mitogen activation.

^bData represent mean \pm standard deviation of absorbance values determined by ChIFN γ specific capture ELISA after *ex vivo* mitogen activation of splenocytes.

*Means \pm standard deviations differ significantly from the negative group value ($P < 0.05$).

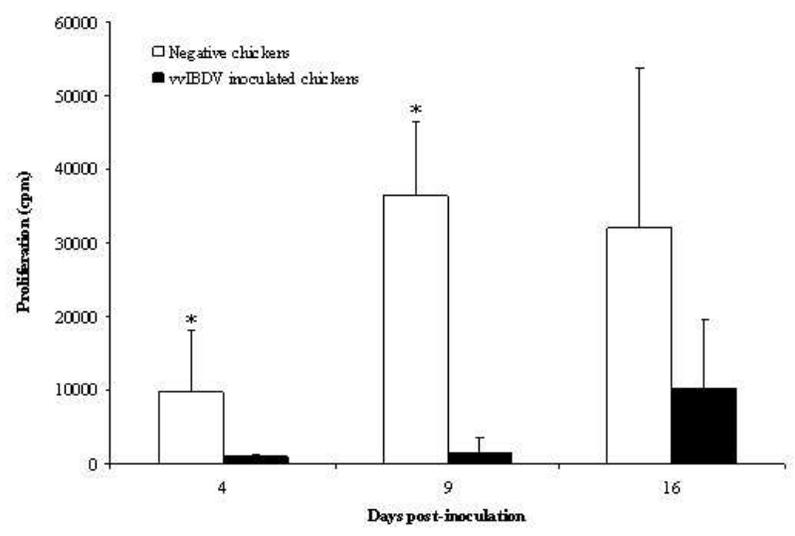
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Table 3 : Proliferation of splenocytes and PBL from SPF chickens after *ex vivo* mitogenic activation in presence of rec-ChIFN γ in culture medium

Mitogen-activated cells	ProEx-ChIFN γ			Irrelevant ProEx-protein (control)	Medium (control)
	Anti-ChIFN γ mAb	Irrelevant mAb			
Splenocytes	22456 \pm 1070 ^B	247 \pm 238 ^C	253 \pm 200 ^C	37904 \pm 674 ^A	37738 \pm 743 ^A
PBL	10746 \pm 921 ^B	132 \pm 125 ^C	148 \pm 97 ^C	16989 \pm 892 ^A	17738 \pm 801 ^A

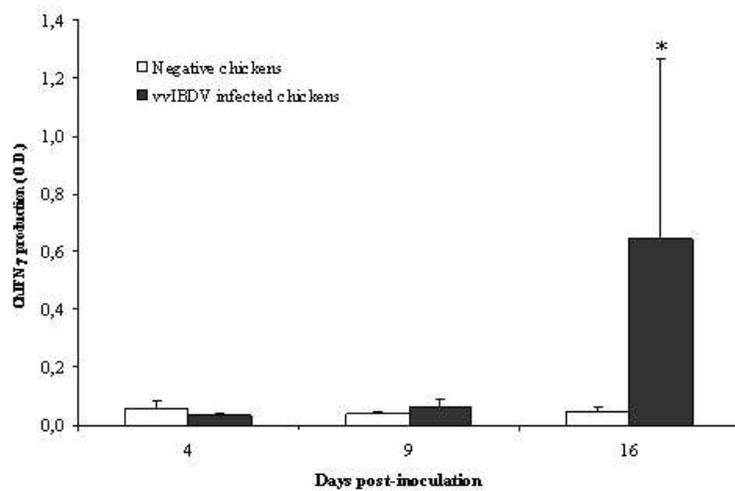
Data represent mean \pm standard deviation of count per minute values determined by proliferation of splenocytes and PBL after *ex vivo* mitogenic activation with PMA/Ionomycin (0.1/0.1 and 1/1 μ g/ml ratio respectively).

Means \pm standard deviations with no common superscript differ significantly ($P < 0.05$).



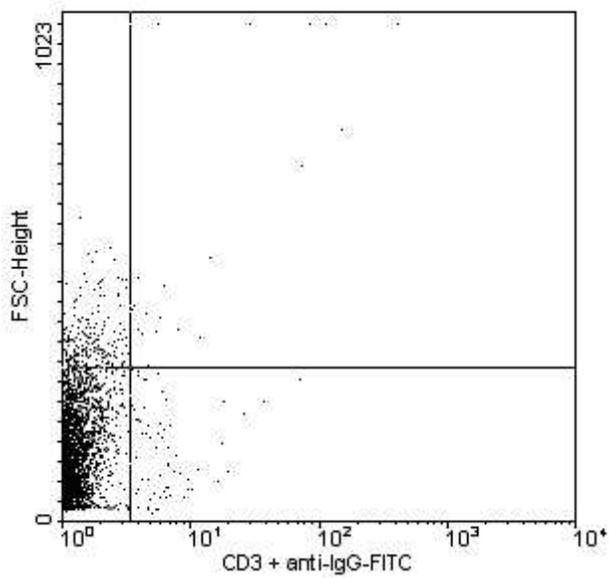
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