

# IFN $\alpha$ and IFN $\gamma$ Impede Marek's Disease Progression

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**Abstract:** Marek's disease virus (MDV) is an alphaherpesvirus that causes Marek's disease, a malignant lymphoproliferative disease of domestic chickens. While MDV vaccines protect animals from clinical disease, they do not provide sterilizing immunity and allow field strains to circulate and evolve in vaccinated flocks. Therefore, there is a need for improved vaccines and for a better understanding of innate and adaptive immune responses against MDV infections. Interferons (IFNs) play important roles in the innate immune defenses against viruses and induce upregulation of a cellular antiviral state. In this report, we quantified the potent antiviral effect of IFN $\alpha$  and IFN $\gamma$  against MDV infections *in vitro*. Moreover, we demonstrate that both cytokines can delay Marek's disease onset and progression *in vivo*. Additionally, blocking of endogenous IFN $\alpha$  using a specific monoclonal antibody, in turn, accelerated disease. In summary, our data reveal the effects of IFN $\alpha$  and IFN $\gamma$  on MDV infection and improve our understanding of innate immune responses against this oncogenic virus.

**Keywords:** Marek's disease virus; recombinant interferons; chicken cytokines; innate immunity; antiviral host defense; antitumor immune response

## 1. Introduction

The highly oncogenic Marek's disease virus (MDV) infects chickens and is a major burden for poultry farming worldwide. Despite the widespread use of vaccines, MDV remains a serious threat to poultry and causes substantial economic losses worldwide every year [1]. This lymphotropic alphaherpesvirus replicates in different immune cell types such as B and T cells [2] and can establish a latent infection of CD4+ T cells which is a prerequisite for malignant transformation of these cells [3,4]. Clinical signs of Marek's disease in chickens include torticollis, ataxia, and paralysis of the legs and wings due to an enlargement of peripheral nerves [5]. In susceptible birds, MDV infection results in the formation of deadly T cell lymphomas in up to 100% of the animals [3].

Interferons (IFNs) are cytokines that possess strong antiviral properties and are a major component of the innate antiviral host defense. They can be divided into type I (IFN $\alpha$  and IFN $\beta$ ), type II (IFN $\gamma$ ), and type III (IFN $\lambda$ ) IFNs, based on their structural and functional features [6]. While type I IFNs are secreted by many different cell types, IFN $\gamma$  is predominantly produced by T helper 1 cells and natural killer cells [6]. The roles of IFN $\lambda$  remain poorly understood [7,8]. It has been shown that IFNs are expressed as an antiviral response to MDV infections *in vitro* [9,10] and in infected chickens [11–16]. Interestingly, a study by Jarosinski et al. describes that oral administration of IFN $\alpha$  reduces MDV replication in experimentally infected chickens, while no data on disease onset and progression are

available [17]. The major aim of this study was to assess the effect of IFNs on MDV replication properties *in vitro* and to determine if recombinant chicken IFN $\alpha$  and IFN $\gamma$  could impair disease onset and progression in chickens and thereby further elucidate the roles of these cytokines in MDV infections.

With this report, we could demonstrate that (i) IFN $\alpha$  and IFN $\gamma$  inhibit MDV *in vitro* replication in a dose-dependent manner, that (ii) the antiviral response of primary chicken fibroblasts on MDV infection is IFN $\alpha$ -mediated, and that (iii) IFN $\alpha$  and IFN $\gamma$  significantly impair disease progression in infected animals.

## 2. Materials and Methods

### 2.1. Cells and Viruses

Chicken embryo cells (CEC) were prepared from specific-pathogen-free (SPF) chicken embryos (Valo BioMedia; Osterholz-Scharmbeck, Germany) that were incubated in-house. CEC were cultured in minimum essential medium (MEM, PAN Biotech; Aidenbach, Germany) supplemented with 1–10% fetal calf serum (FCS; PAN Biotech) and 1% antibiotics (100 U/mL penicillin and 100  $\mu$ g/mL streptomycin; AppliChem; Darmstadt, Germany) at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> [18]. B cells were obtained as previously described and cultured in Iscove's basal medium with 8% FCS, 2% chicken serum, and penicillin/streptomycin and activated with recombinant chCD40L [19–21]. For *in vitro* assays, we used the very virulent RB-1B strain [22] and the vaccine strain CVI988 [23]. The very virulent plus Italian MDV-1 strain EU-1 [24] was used in both animal experiments.

### 2.2. Chicken Interferons and Antibodies

Recombinant chicken IFN $\alpha$  (rChIFN $\alpha$ ) and recombinant chicken IFN $\gamma$  (rChIFN $\gamma$ ) were produced in *Escherichia coli* [25,26] and protein concentrations were determined using the Bradford assay [25,27]. Polyclonal rabbit anti-IFN $\alpha$  and polyclonal rabbit anti-IFN $\beta$  were obtained as previously described [25,28] and the monoclonal antibody (mAb 8A9) against chicken IFN $\alpha$  was obtained from a rat as reported earlier [29].

### 2.3. In Vitro Assays

Replication properties and cell-to-cell spread of the MDV strain RB-1B in CEC were determined by plaque size assays in the presence or absence of the IFNs in three independent biological replicates as previously described [30]. MDV infection in primary chicken B cells was assessed by flow cytometry as previously described [20]. B cells were infected with RB-1B by co-cultivation with infected CEC in the presence and absence of IFN $\alpha$ . To test the antiviral activity of MDV-infected cell culture supernatants, we used the vesicular stomatitis Indiana virus (VSV) bioassay as described by Lewis [31–33] (Figure S1). Briefly, VSV replication is highly susceptible to IFNs, a property used to assess the presence of IFNs in cell culture supernatants. Supernatants of MDV-infected CEC were added to a monolayer of CEC-32 cells and infected with VSV. Cells were stained with neutral red, washed with phosphate-buffered saline (PBS), lysed, and the optical density measured at 540 nm. The amounts of viable cells protected by IFNs corresponds to the optical density [31,33]. To confirm the specificity, the antiviral activity could be blocked by addition of anti-IFN $\alpha$  and anti-IFN $\beta$  antisera or an anti-IFN $\alpha$  mAb (Figure S1).

### 2.4. Ethics Statement and Animal Experiments

This report describes two animal studies. In both, SPF Lohmann selected leghorn (LSL; Lohmann Animal Health, Cuxhaven, Germany) were hatched and kept under SPF conditions. The studies were approved by the responsible authority (the Animal Research Board of the State of Lower Saxony, Germany; animal use protocol # 295/01 (IFN $\alpha$ ) and 45/01 (IFN $\gamma$ )) and were conducted according to relevant national and international guidelines for the humane use of animals. Chickens had *ad libitum* access to food and water, and were routinely checked for clinical signs like ataxia, ruffled feathers, and somnolence throughout the 70 day experiments.

In the first animal study, 15 chickens per treatment group (1: mock/PBS, 2: IFN $\alpha$  (250 IU), 3: anti-IFN $\alpha$  mAb (100  $\mu$ g)) were injected intramuscularly with a low dose of the very virulent + (vv+) strain EU-1 at 2 days post-hatching (100  $\mu$ L lymphocyte suspension). Treatments with IFN $\alpha$  and anti-IFN $\alpha$  mAb were administered intraperitoneally: The first treatment was given at 1 day post-hatching and all chickens received repeated treatment every third day over the period of 10 weeks.

In the second animal experiment, seven chickens per group (1: mock/PBS, 2: IFN $\gamma$  (200 IU)) were intramuscularly infected with a high dose of the vv+ strain EU-1 (200  $\mu$ L lymphocyte suspension) at 2 days post-hatching. Treatments were administered intraperitoneally: The first treatment was given at 1 day post-hatching and all chickens received repeated treatment every third day.

The successful establishment of infection was confirmed in blood samples of three animals per group by PCR detecting the infected cell protein 4 (ICP4) gene of MDV (Tables S1 and S2) [34]. All chickens were humanely euthanized and examined post-mortem for tumor lesions once clinical symptoms appeared or upon the termination of the experiment. Tumors were mainly detected in the visceral organs (spleen, liver, and kidneys).

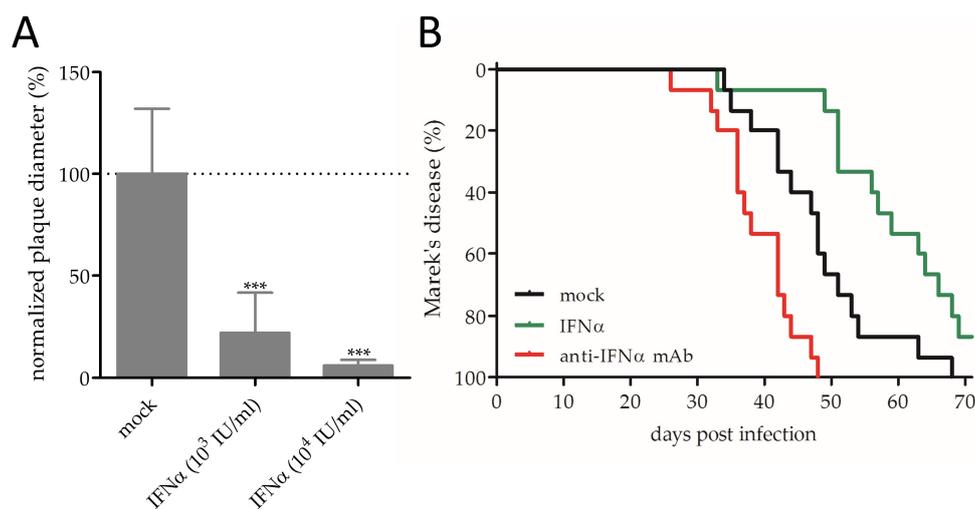
### 2.5. Statistical Analyses

Statistical analyses were performed with Graph-Pad Prism v5. One-way analysis of variance (ANOVA) with Bonferroni correction on multiple comparisons was used for plaque size assays and the effect of IFN on the infection of primary chicken B cells. Kaplan–Meier survival analysis along with the Mantel–Cox test (log-rank test) was used for analyses of the animal experiment data. Data were considered significantly different if  $p \leq 0.05$ .

## 3. Results and Discussion

### 3.1. Effect of IFN $\alpha$ on MDV Replication and Pathogenesis

To assess the effect of recombinant IFN $\alpha$  on MDV replication in vitro, we performed plaque size assays and observed a dose-dependent effect on virus replication in CEC (Figure 1A) and in primary chicken B cells (Figure S2). This is consistent with previously described reduction in the plaque numbers upon IFN $\alpha$  treatment of cells [17,35].



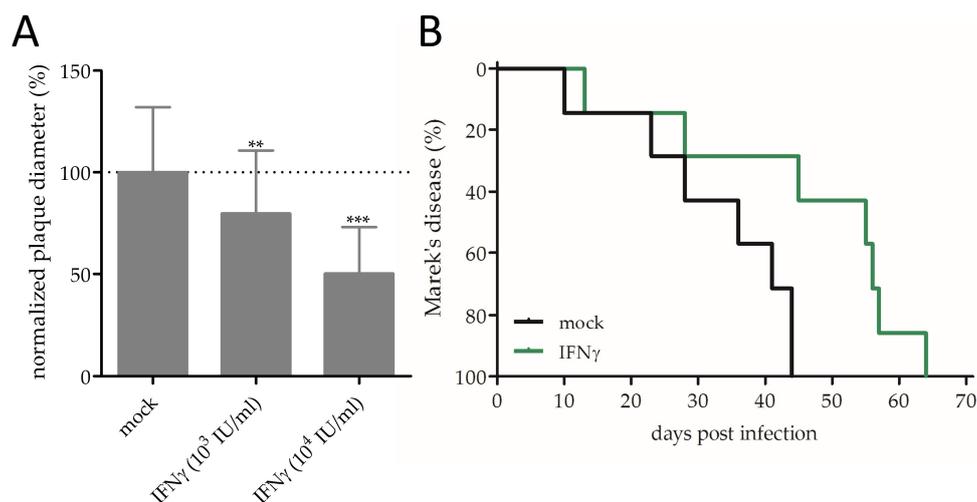
**Figure 1.** Anti-Marek's disease virus (MDV) effects of interferon-alpha (IFN $\alpha$ ): (A) Dose-dependent replication inhibition upon IFN $\alpha$  treatment, as assessed by conventional plaque size assays (\*\*\*)  $p < 0.001$ , one-way ANOVA with Bonferroni correction). (B) Kaplan–Meier analysis of Marek's disease incidence in chickens with indicated treatment (Mantel–Cox test;  $p < 0.001$ ).

Moreover, we could demonstrate that IFN $\alpha$ , but not IFN $\beta$ , was released into the supernatant upon infection of primary CEC with MDV (Figure S1). In line with this, it has recently been shown that the MDV-encoded protein RLORF4 inhibits IFN $\beta$  production in chicken fibroblasts [36]. These data confirm the IFN $\alpha$ -mediated antiviral response on the protein level—a response that has so far only been shown on the RNA level [9]. In experimentally infected chickens that were treated with IFN $\alpha$ , treatment significantly delayed the disease incidence with median survival rates of mock and IFN $\alpha$  treated animals of 48 and 59 days respectively (Figure 1B;  $p < 0.05$ ). These findings indicate that administration of IFN $\alpha$  has a protective effect, but that it cannot prevent disease in MDV-infected chickens. This could be due to the lower efficacy of IFN $\alpha$  in inhibiting MDV infection in primary chicken B cells (Figure S2). In those cells, high concentrations of IFN $\alpha$  only reduced MDV infection to 54% and 49% ( $\pm 12.1$  standard deviation) 24 and 48 hours post-infection, respectively, indicating that the potency of IFN $\alpha$  to suppress MDV replication substantially differs in different cell types.

Furthermore, we could demonstrate that treatment with anti-IFN $\alpha$  mAb accelerates the onset of disease, highlighting the important role of early IFN $\alpha$  responses. The median survival was reduced to 38 days and it took approximately 3 weeks less until all animals showed clinical signs of Marek's disease (Figure 1B;  $p < 0.05$ ). Taken together, we could demonstrate that IFN $\alpha$  impairs MDV replication in vitro and that it extends the survival of treated chickens. Moreover, the important role of IFN $\alpha$  in MDV infection was confirmed by blocking endogenous IFN $\alpha$  with mAb.

### 3.2. Effect of IFN $\gamma$ on MDV Replication and Pathogenesis

Similar to IFN $\alpha$ , we also observed a dose-dependent inhibition of MDV replication upon IFN $\gamma$  treatment in vitro (Figure 2A). This data is consistent with previously described IFN $\gamma$ -dependent reduction in the plaque numbers for different MDV strains and turkey herpesvirus [37]. Interestingly, the effect of treatment was less pronounced when compared to IFN $\alpha$  treatment. Both IFNs are known to shape the initial inflammatory and downstream adaptive immune responses. In addition, it is likely that direct antiviral effects of IFN $\gamma$  are broader and less specific due to its role in the upregulation of inducible nitric oxide synthase expression [38] and other immunomodulatory functions. IFN $\alpha$ , on the other hand, is known to be one of the first cytokines produced during a virus infection and induces an immediate induction of antiviral interferon-stimulated genes.



**Figure 2.** Anti-MDV effects of interferon-gamma (IFN $\gamma$ ): (A) Dose-dependent replication inhibition upon IFN $\gamma$  treatment, as assessed by conventional plaque size assays (\*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ , one-way ANOVA with Bonferroni correction). (B) Kaplan–Meier analysis of Marek's disease incidence in chickens with indicated treatment (Mantel–Cox test;  $p = 0.0226$ ).

Notably, treatment of experimentally infected chickens with IFN $\gamma$  also led to a delay of disease progression (Figure 2B;  $p < 0.05$ ). Here, we observed a median survival of 36 days (mock) and 55 days (IFN $\gamma$ ). It has been shown that MDV tumor tissue contains elevated levels of interferon-induced proteins, amongst them IFN $\gamma$ -inducible protein 30 (IFI30), which has been discussed to possess antitumor properties [39,40]. Hence, IFN $\gamma$  could not only inhibit MDV replication, but also induce an antitumor response [41]. The observed differences in the median survival rates of the mock groups in the IFN $\alpha$  and IFN $\gamma$  in vivo experiments could be explained by the different doses of MDV infections (see Section 2.4).

#### 4. Conclusions

Collectively, these data show that interferons efficiently inhibit MDV replication and significantly delay Marek's disease progression. Nevertheless, treatment with IFN $\alpha$  and IFN $\gamma$  alone did not prevent disease and tumor formation in our studies. It remains unclear how MDV circumvents IFN-mediated host immune responses and how this oncogenic alphaherpesvirus could alter IFN production to successfully infect, replicate, shed, and cause clinical disease. The observed discrepancy between in vitro and in vivo results could be explained by the observed differences in the efficacy of IFN treatment on MDV replication in different cell types in vitro with less pronounced antiviral activity in B cells, the primary target cells for MDV replication. Future research could focus on synergistic effects of IFN $\alpha$  and IFN $\gamma$  [42] or the use of recombinant chicken IFNs in vaccine formulations to boost the chicken immune system to ward off viral diseases [43–45]. Beyond type I and II IFNs, the role of type III IFNs should be investigated in the context of MDV infection at mucosal surfaces. Finally, novel methodology in avian immunology and infectious diseases research such as genetically modified chickens [46] or the increasing availability of avian cell markers as well as in vitro and in vivo tools [47] will certainly improve our understanding of these processes in the near future.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/1999-4915/11/12/1103/s1>, Figure S1: Antiviral activity of chicken embryo cells (CEC) culture supernatant; Figure S2: IFN $\alpha$ -mediated MDV replication inhibition in primary chicken B cells; Table S1: Primers used for the ICP4 PCR; Table S2: PCR confirmation of MDV infections in animals.

**Author Contributions:** Conceptualization, F.F., B.B.K., and B.K.; formal analysis, L.D.B. and O.H.; investigation, L.D.B., O.H., S.H., F.F., and T.V.; data curation, L.D.B. and O.H.; writing—original draft preparation L.D.B.; writing—review and editing, L.D.B., O.H., S.H., F.F., T.V., B.B.K., and B.K.; visualization, L.D.B.; supervision, F.F., B.B.K., and B.K.; project administration, B.B.K. and B.K.; funding acquisition, F.F., B.B.K., and B.K.

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**Conflicts of Interest:** The authors declare no conflict of interest regarding the publication of this article.

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