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CpG and poly(I:C) stimulation of dendritic cells and fibroblasts limits herpes simplex virus type 1 infection in an IFNβ-dependent and -independent way

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ABSTRACT

Viral activation of toll-like receptors (TLRs) on dendritic cells (DCs) leads to production of various cytokines, including antiviral type I interferons (IFNs). Synthetic ligands specific for TLRs are also able to induce the production of type I IFNs (IFNα/β) by DCs, suggesting that these ligands have potential as antiviral drugs. In this *in vitro* study we extensively investigated the antiviral activity of various TLR ligands. Mouse bone marrow (BM) cells were differentiated into plasmacytoid and conventional DCs (pDCs and cDCs), stimulated with various TLR ligands and tested the antiviral abilities of collected supernatants in an *in vitro* herpes simplex virus type 1 (HSV-1) infection model. We observed a significant IFNβ-, (but not IFNα-) dependent reduction in HSV-1 infection when a mixed pDC/cDC population was stimulated with the TLR9 ligand CpG. In the absence of pDCs, TLR stimulation resulted in less pronounced antiviral effects. The most pronounced antiviral effect was observed when both DC subsets were stimulated with poly(I:C). A similar noticeable antiviral effect was observed when fibroblasts (L929 cells) were stimulated directly with poly(I:C). These poly(I:C)-mediated antiviral effects were only partially IFNβ-mediated and probably TLR independent. These data demonstrate that TLR ligands are not only able to produce type I IFN but can indeed act as antiviral drugs. In particular poly(I:C), which exerts its antiviral effects even in the absence of DCs, may become a promising drug e.g. to prevent respiratory infections by topical intranasal application.

1. Introduction

Type I interferons (IFNs) are the key cytokines produced predominantly by innate immune cells to combat viral infections. After viral recognition the release of IFN induces the expression of so-called interferon stimulated genes (ISGs) which subsequently activate a variety of antiviral processes including amplification of IFN signalling and the activation of adaptive immunity (Borden et al., 2007; Der et al., 1998; Fitzgerald-Bocarsly and Feng, 2007; Sadler and Williams, 2008). This will ultimately result in the induction of a non-virus-specific antiviral state in infected cells, culminating in direct inhibition of viral replication while also enhancing the host's specific antiviral immune responses through IFN-related immuno-modulatory stimuli.

Because of this strong antiviral activity, type I IFNs have also been used in clinical practice. For example, pegylated interferon alpha (Peg-IFNα) in combination with ribavirin is currently recommended as standard-of-care treatment of chronic hepatitis C virus infection. However, depending on the HCV genotype involved, success rates of Peg-IFNα/ribavirin treatment vary significantly. Moreover, in clinical practice approximately 10–15% of patients discontinue this therapy due to adverse effects which impacts most, if not all, organ systems (McHutchison et al., 2009; Rustgi, 2010; Sulkowski et al., 2011). Therefore, there is need for alternative therapies.

Although a large variety of immune as well as non-immune cells is able to produce type I IFNs, the most important cells in this respect are dendritic cells. In particular plasmacytoid, but also conventional dendritic cells are well able to produce significant amounts of type I IFNs (IFNαβ). The type I interferon response is usually initiated following recognition of viral components by pathogen recognition receptors e.g. toll-like receptors (TLRs) and

Abbreviations: BM, bone marrow; BM-DC, BM-derived DC; cDC, conventional dendritic cell; CPE, cytopathogenic effect; DC, dendritic cell; FL, Flt-3L; GM, GM-CSF; HSV, herpes simplex virus; IFN, interferon; LPS, lipopolysaccharide; MCMV, mouse cytomegalovirus; MDA5, melanoma-differentiation-associated gene 5; ODN, oligodeoxynucleotides; pDC, plasmacytoid dendritic cell; PRR, pattern recognition receptor; TLR, toll-like receptor; UNG, uracil-N-glycosylase.

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cytoplasmic receptors (Bowie and Unterholzner, 2008; Haller et al., 2006; Kato et al., 2006; Katze et al., 2008; Trinchieri and Sher, 2007). The plasmacytoid DC (pDC) senses single-stranded RNA (ssRNA) and CpG DNA from viruses via TLR7 and 9, respectively (Barchet et al., 2005; Blasius and Beutler, 2010; Fitzgerald-Bocarsly et al., 2008; Fitzgerald-Bocarsly and Feng, 2007; Ito et al., 2006). Conventional DC (cDCs), on the other hand, sense the viral intermediate double-stranded RNA (dsRNA) via TLR3 (Baig and Fish, 2008; Blasius and Beutler, 2010; Fitzgerald-Bocarsly and Feng, 2007). Next to these TLRs it has previously been shown that also TLR2 and 4, located on human monocytes, are involved in viral detection (Compton et al., 2003; Kawai and Akira, 2010; Kurt-Jones et al., 2000, 2004). Cytoplasmic receptors, on the other hand, are present in almost all cell types and recognize dsRNA during viral replication (Kawai and Akira, 2010; Yoneyama and Fujita, 2009).

As DCs express a specific repertoire of TLRs, it has been suggested that synthetic TLR ligands, targeting these receptors, may have a therapeutic potential as antiviral compounds. Yet, although it is well known that some TLR ligands are well able to initiate the release of type I interferons by DCs (Brawand et al., 2002; Coccia et al., 2004; Gautier et al., 2005; Gibson et al., 2002; Lore et al., 2003), the direct antiviral effects of TLR-mediated DC activation have been studied less intensively. Therefore, in this study the antiviral potency of various TLR ligands was examined in an *in vitro* model of herpes simplex virus 1 (HSV-1) infection.

We found that in particular IFNβ (but not IFNα), produced in large amounts by the CpG- and poly(I:C)-stimulated mixed DC population, was very effective in limiting HSV replication. Furthermore, L929 fibroblasts also induced an antiviral response when stimulated with poly(I:C). This response was, however, only partially IFNβ-mediated and suggests the importance of other antiviral pathways.

2. Materials and methods

2.1. Mice

Bone marrow was derived from male BALB/c mice (8–14 weeks of age), obtained from Charles River Laboratories and maintained under normal conditions. Mice were euthanized by intraperitoneal injection of Nembutal® (150 mg/kg, Sanofi Sante B.V., Maassluis, the Netherlands). The study was approved by the ethical committee for animal experiments of the Maastricht University.

2.2. Isolation and differentiation of bone marrow cells

Femur and tibia were removed and flushed with RPMI 1640 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% FCS (Lonza, Verviers, Belgium) and 40 μg/ml gentamycin (Centrafarm, Etten-Leur, the Netherlands) to obtain the BM cells. BM cells were resuspended in NH_4Cl buffer containing EDTA and incubated for 10 min on ice to lyse red blood cells.

BM cells were cultured in 24-well tissue culture plates (Becton Dickinson, NJ, USA) at 10^6 cells/ml in RPMI 1640 medium (Invitrogen, Grand Island, NY, USA) with 10% FCS (Lonza, Verviers, Belgium) and 40 μg/ml gentamycin (Centrafarm, Etten-Leur, the Netherlands). The medium was supplemented with either 200 ng/ml human Flt-3L (Miltenyi Biotec, Leiden, the Netherlands) or 20 ng/ml GM-CSF (Miltenyi Biotec, Leiden, the Netherlands) for differentiation into pDCs and cDCs or cDCs only, respectively. When GM-CSF was used, the medium was refreshed 3 and 6 days after seeding the cells in the plates. Cells were allowed to differentiate for 8 days at 37 °C and 5% CO_2 before stimulation with different TLR ligands was started.

2.3. TLR ligands and stimulation

Lipopolysaccharide (LPS) (*Escherichia coli serotype 055:B5(L2880)*) was obtained from Sigma (St Louis, USA). R-848, poly(I:C) LMW and CpG ODN 1585 were all obtained from Invivogen (San Diego, CA, USA). At day 8, the medium of the DCs was removed and replaced with medium containing different TLR ligands. Differentiated BM cells were stimulated with either LPS (100 ng/ml), R-848 (1 and 10 μg/ml), poly(I:C) (50 μg/ml) or CpG ODN 1585 (0.1 and 1 μM) for 24 h at 37 °C and 5% CO_2. Afterwards, cells were snap-frozen in liquid nitrogen and stored at −80 °C for future gene expression analysis. Supernatants of the stimulated DCs were stored at −20 °C until further use.

2.4. Cells and virus

L929 cells (ATCC CCL-1) (Rockville, MD, USA) were cultured in Earle's Minimal essential medium (EMEM) (Invitrogen, Grand Island, NY, USA) supplemented with non-essential amino acids (MP Biomedicals, Solon, Ohio, USA), L-glutamine (2 mmol/L), sodium pyruvate (1 mmol/L) and 10% FCS (Lonza, Verviers, Belgium). Cells were allowed to grow in T75 flasks at 37 °C and 5% CO_2.

HSV-1 was obtained from ATCC (VR-539) and was propagated in Vero cells (ATCC CCL-81) (Rockville, MD, USA) in EMEM (Invitrogen, Grand Island, NY, USA) with 2% FCS (Lonza, Verviers, Belgium), non-essential amino acids (MP Biomedicals, Solon, Ohio, USA), L-glutamine (2 mmol/L) and sodium pyruvate (1 mmol/L). When 100% cytopathogenic effect (CPE) was achieved, cell debris was removed by centrifugation and viral titers in the supernatant were determined by plaque assay.

2.5. Stimulation and infection protocol

To test the antiviral potency of the conditioned media obtained from TLR-stimulated DCs, L929 cells were grown in 24-well tissue culture plates (Becton Dickinson, NJ, USA) until confluency and, after washing with PBS, were exposed to the collected supernatants. After 18 h incubation, the conditioned medium was removed, cells were washed with PBS and infected with HSV-1 (MOI 0.1) in EMEM medium without FCS for 1 h at 37 °C and 5% CO_2. Next, the HSV-1-containing supernatant was removed and replaced by normal EMEM medium without FCS. The cells were harvested 30 h post infection (p.i.), snap-frozen in liquid nitrogen and stored at −80 °C for DNA and RNA isolations. The supernatant was stored at −80 °C until used for plaque assay.

2.6. Quantitive PCR (qPCR)

DNA was extracted from frozen cell pellets according to the Wizard® Genomic DNA Purification Kit (Promega Benelux B.V., Leiden, the Netherlands) according to the manufacturer's instructions. DNA purity and quantity were measured with the Nanondrop® ND-1000. The DNA isolates were amplified in a volume of 25 μl containing 12,5 μl IQ™ Sybr green mix (Bio-Rad, Hercules, CA, USA), HSV-1 forward and reverse primer, and DNA sample. HSV-1 was detected by using a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Thermal cycling was started with uracil-N-glycosylase (UNG) activation for 2 min at 50 °C, followed by HotStarTaq activation during 15 min at 95 °C. Subsequently, 40 cycles of amplification were run consisting of 15 s at 95 °C (denaturation) and 1 min at 60 °C (annealing and attaching).

To determine the actual number of HSV-1 DNA copies, a DNA standard curve was used. Dilutions were made from a plasmid, which contains the HSV-1 PCR-target sequence. Concentrations used ranged from 10^7 to 10^0 copies, with a dilution factor of 10.

Copy numbers were quantified by the standard curve using the iQ™5 version 2.0 Optical System Software.

2.7. RT-qPCR

RNA was isolated from frozen cell pellets with the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Remaining DNA was removed by DNAse treatment (Turbo DNA-free™ kit, Ambion, Austin, TX, USA). Subsequently, RNA was reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). qPCR was performed as described above. Primer sets used are listed in Supplementary Table 1. To control for DNA contamination, in every PCR run a sample was included which was not reverse transcribed. Relative expressions were determined by using the $2^{-\Delta Ct}$ (Schmittgen and Livak, 2008) method, normalized to GAPDH values. All samples were measured in duplicate.

2.8. Plaque assay

To determine the presence of infectious HSV-1 particles, collected supernatant from infected L929 cells was added to Vero cells (ATCC CCL-81) (Rockville, MD, USA) grown until confluency in 24-well plates. After 1 h at 37 °C and 5% CO_2, supernatant was removed and, after the cells had been washed with PBS, replaced by a medium-agarose mixture (1:1). This EMEM medium without phenol red (Invitrogen, Grand Island, NY, USA) was supplemented with 2% FCS (Lonza, Verviers, Belgium), non-essential amino acids (MP Biomedicals, Solon, Ohio, USA), sodium pyruvate (1 mmol/L) and L-glutamine. After 72 h incubation at 37 °C and 5% CO_2 cells were exposed to a 3.7% formaldehyde solution for 4 h and afterwards stained with 1% methylene blue.

2.9. Neutralization assay

Pure or diluted (5× or 50×) supernatant from stimulated DCs was incubated with IFNβ-antibody (2×10^3 units/ml; PBL Biomedical Laboratories, NJ, USA) for 1 h at 37 °C and 5% CO_2 to neutralize IFNβ. L929 cells were seeded and allowed to grow until confluency in 96-well plates. These L929 cells were then incubated with the conditioned, +/− antibody-containing supernatants for 18 h and were subsequently infected with HSV-1 according to the infection protocol and viral copies were determined by qPCR.

2.10. Detection of IFNβ production

An enzyme-linked immunosorbent assay (PBL Biomedical Laboratories, NJ, USA) was used to detect IFNβ in the supernatant of stimulated DCs.

2.11. Statistical analysis

The Student's *t*-test was used to analyze differences between control and stimulated samples. Differences between multiple groups were determined by one-way ANOVA with a Bonferroni post hoc test. Values of $p < 0.05$ were considered statistically significant. Data are expressed as mean ± SEM, unless stated otherwise.

3. Results

3.1. Supernatant of TLR-stimulated DCs limits viral infection of L929 cells

As illustrated in Fig. 1A, HSV-1 infection of L929 cells could be inhibited significantly when cells were pre-treated for 18 h with conditioned supernatants collected from Flt-3L (FL) BM-DC cultures stimulated with either LPS, poly(I:C) or CpG (1 μM) for 24 h. No reduction in viral copy number was observed when supernatants of R-848-stimulated FL BM-DC cultures were used. Likewise, poly(I:C) stimulation of GM-CSF (GM) BM-DC cultures resulted in a strong reduction of viral DNA copies, while viral copy numbers were also reduced following pre-treatment with either LPS or CpG (1 μM) (Fig. 1B). Moreover, also R-848 induced a small, though significant decrease in viral copy numbers.

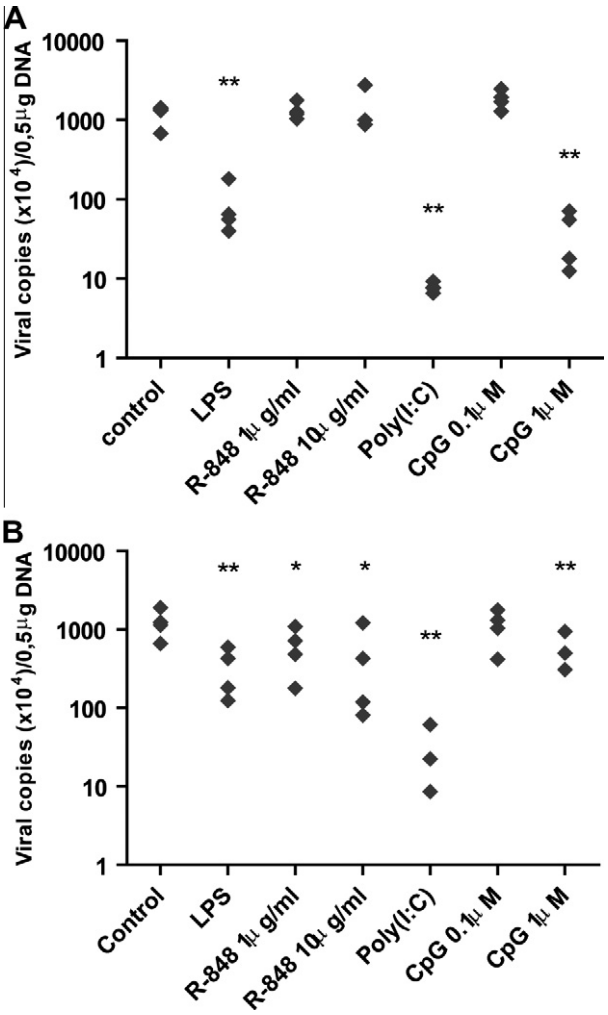

Fig. 1. Differential reduction of HSV-1 DNA levels by TLR ligands. Antiviral effect of supernatant from TLR stimulated FL BM-DCs (A) or GM BM-DCs (B) on L929 cells subsequently infected with HSV-1. Symbols indicate BM-DC supernatant from individual mice ($n = 4$). Viral copies were determined by qPCR. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus control.

The inhibition of viral replication by the TLR ligands was also determined at the mRNA levels. In accordance with the reduction in DNA copy numbers (Fig. 1), HSV-1 mRNA expression was significantly reduced in L929 cells treated with conditioned medium from either LPS-, poly(I:C)- or CpG-stimulated FL BM-DCs (Fig. 2A). In contrast, only conditioned medium from LPS- or poly(I:C)-stimulated GM BM-DCs significantly reduced HSV-1 mRNA, while no effect was observed when conditioned medium from GM BM-DC stimulated with CpG was used (Fig. 2B). Moreover, the earlier observed R-848-induced reduction in viral copies DNA copies was at the mRNA level only confirmed after stimulation with 1 μg/ml (but not 10 μg/ml) R-848. Overall, our data showed pronounced antiviral effects of poly(I:C), CpG and LPS, while the effects of R848 were rather limited.

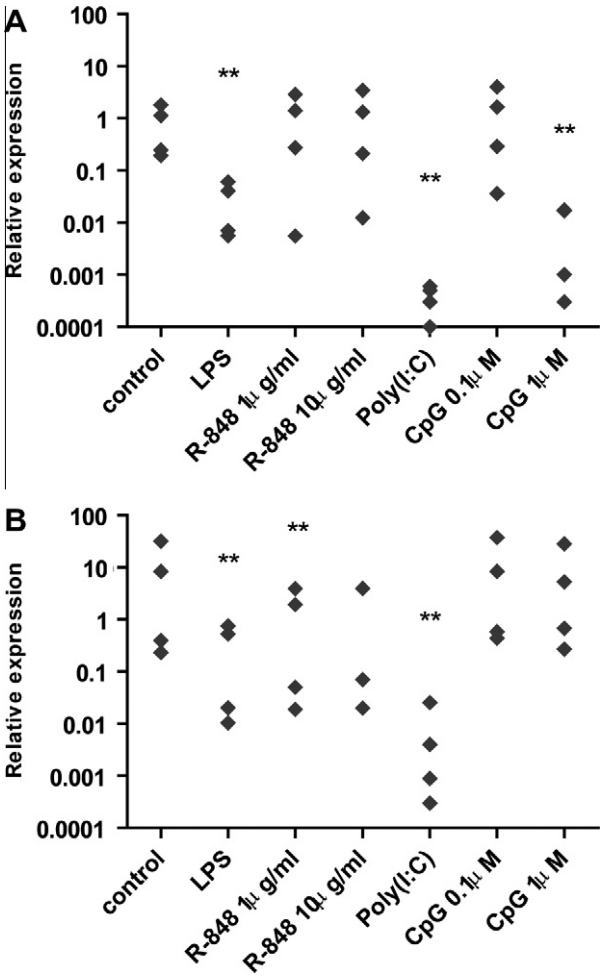

Fig. 2. Differential reduction of HSV-1 mRNA levels by TLR ligands. Effect of supernatant from TLR stimulated FL BM-DCs (A) or GM BM-DCs (B) on L929 cells subsequently infected with HSV-1. HSV-1 is displayed as relative expression compared to GAPDH values. Symbols indicate BM-DC supernatant from individual mice ($n = 4$). $^{**}P < 0.01$ and $^{*}P < 0.05$ versus control.

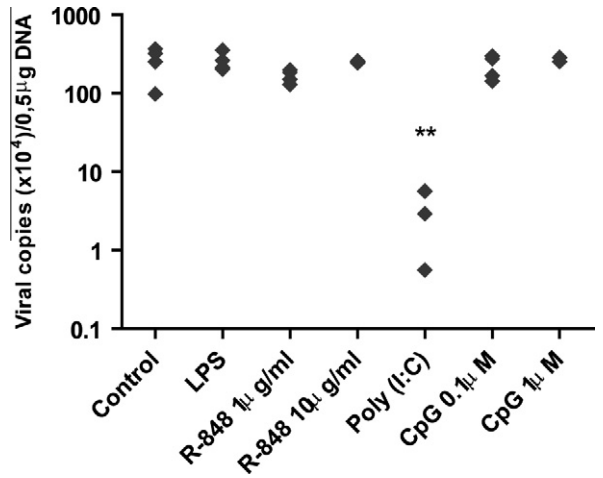

Fig. 3. HSV-1 inhibition by TLR ligands requires BM-DCs. TLR ligands were directly administered to L929 cells. Symbols indicate independent experiments ($n = 4$). Viral copies were determined by qPCR. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus control.

To control for antiviral effects due to TLR ligands still present in the supernatant of stimulated DCs, TLR ligands were administered directly to L929 cells 18 h before infection (Fig. 3). Except for poly(I:C), no reduction in HSV-1 copies was observed. This indicates that the observed antiviral effect of TLR ligands is dependent on stimulation of BM-DCs, with the exception of poly(I:C), which significantly reduced viral copies in L929 cells independent of the BM-DCs.

Next, the potency of various TLR ligands to prevent the formation of infectious HSV-1 virus particles was analyzed with a plaque assay. After administration of conditioned BM-DC supernatants to the L929 cells and subsequent infection with HSV-1 for 30 h, the presence of infectious particles in the supernatants of infected L929 cells was examined. Vero cells were incubated for 1 h with the L929 supernatant and plaque formation was determined after 72 h. Again, the degree of plaque formation corresponded with the results at DNA and mRNA levels as the number of plaques were drastically reduced when L929 cells were pre-treated with the conditioned supernatant of LPS-, poly(I:C)- or CpG (1 μM)-stimulated FL BM-DCs (Fig. 4B). Analogous to the DNA and mRNA data, limited reduction of plaque formation was achieved after R-848- and LPS-treatment of GM BM-DCs while stimulation with poly(I:C) was most protective (Fig. 4C). Furthermore, a strong reduction of plaque formation was once more observed when poly(I:C) was directly added to L929 cells thereby demonstrating again that poly(I:C) can induce DC-independent antiviral effects (Fig. 4D).

These data clearly demonstrate that stimulation of DCs with various TLR ligands results in significant antiviral effects. Moreover, FL BM-DCs seem more potent than GM BM-DCs in preventing viral replication and a strong DC-independent antiviral effect of poly(I:C) was observed.

3.2. IFNβ inhibits HSV-1 replication

To investigate the mechanisms involved in the observed antiviral effects, the mRNA expression of IFN-α4 and -β in the differentiated BM-DCs was determined. These cytokines are normally the first antiviral mediators produced in mice during viral infection (Baig and Fish, 2008; Katze et al., 2002). Basal IFNα4 mRNA expression was low in both DC cell cultures. Surprisingly, however, only a slight (non-significant) increase was observed when FL BM-DCs were stimulated with poly(I:C) or CpG (1 μM) (data not shown), which implicates that IFNα4 might play only a minor role in the observed antiviral effect. Yet, in the same samples the expression of IFNβ mRNA was strongly enhanced following LPS, CpG or poly(I:C) stimulation (Fig. 5A). Importantly, the IFNβ expression in these samples correlated with the antiviral effect of these TLR ligands in the HSV-1 infection assay. R-848 (10 μg/ml) resulted in a minor increase in IFNβ expression, which is probably not sufficient for a detectable antiviral effect, as observed in Figs. 2 and 4. In GM BM-DCs, on the other hand, both IFNα4 (data not shown) and IFNβ expression remained very low in all conditions (Fig. 5B).

These results indicate that IFNβ is the most important cytokine in the antiviral effect observed following (FL) BM-DC stimulation with various TLR ligands. To further explore the role of IFNβ we performed neutralization experiments. Therefore, a IFNβ neutralizing antibody was added to the supernatants of LPS-, poly(I:C)- and CpG (1 μM)-stimulated FL BM-DCs. Subsequently, after a 1 h preincubation period at 37 °C these supernatants were added to L929 cells prior to HSV-1 infection. As expected, neutralizing IFNβ significantly reduced the antiviral effect of the conditioned media (Fig. 6A,C,E). In similar experiments using an IFNα neutralizing antibody no effect of the antibody was observed (data not shown), further emphasizing the predominant role of IFNβ in the observed antiviral effect.

Stimulation of GM BM-DCs with poly(I:C), and to a lesser extent R-848 and LPS, also induced an antiviral effect in our HSV-1 infection assay. However, RT-qPCR results demonstrated that the

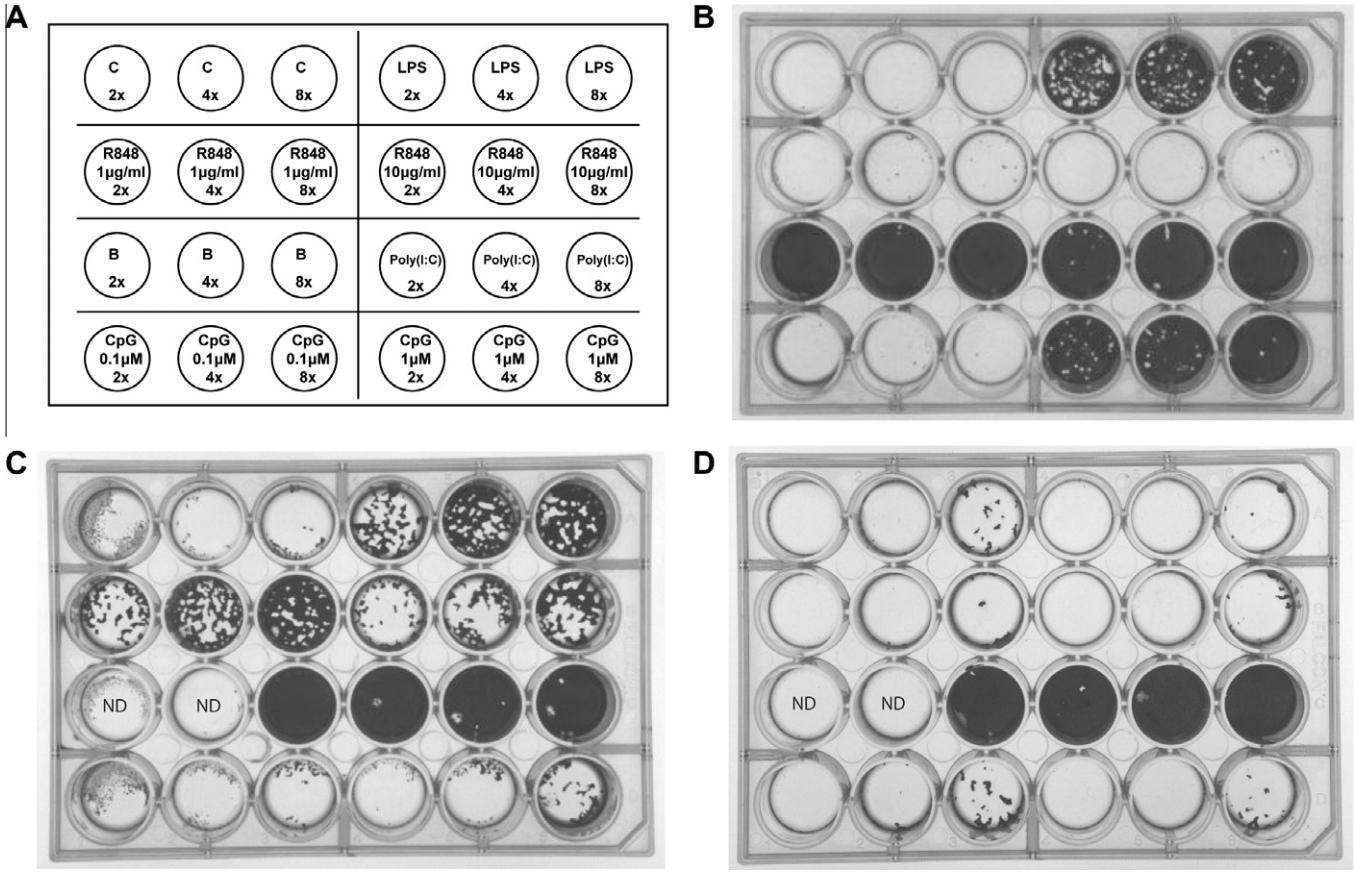

Fig. 4. Differential reduction of infectious HSV-1 by TLR ligands. Vero cells were exposed to supernatant from infected L929 cells after stimulation with supernatant from TLR-stimulated DCs. Schematic figure representing the different samples and dilutions (A). Viral solutions obtained from L929 cells stimulated with supernatant from FL BM-DCs (B), GM BM-DCs (C) and directly stimulated L929 cells (D) show various degrees of infection. Figures are representative of three independent experiments. C = control, B = Blank. ND = not done.

expression of IFNβ mRNA in GM BM-DC is low and not significantly enhanced after stimulation with the respective TLR ligands (Fig. 5B). Despite the low IFNβ mRNA expression, the antiviral effect of the LPS and R848 could be inhibited by the IFNβ neutralizing antibody (Fig. 6B,D,F). The neutralizing effect in poly(I:C)-stimulated GM BM-DCs was less pronounced compared to the effect observed with poly(I:C)-stimulated FL BM-DCs. This suggests that other cytokines than IFNβ may be important in mediating the antiviral effects of poly(I:C).

These expression analyses and neutralization assays suggest that especially in FL BM-DCs, IFNβ is the key cytokine in the TLR-mediated antiviral effect. These results were confirmed by an IFNβ ELISA, which showed exclusive production of IFNβ by FL BM-DCs stimulated with poly(I:C) or CpG (1 μM) (Fig. 7) while the IFNβ production in all other samples was below the detection limit (15.6 pg/ml, data not shown).

3.3. Effect of poly(I:C) on L929 cells

Of all the TLR ligands tested, poly(I:C) was the most potent inhibitor of HSV-1 replication. This antiviral effect seems DC-independent as similar effects were observed when L929 cells were stimulated directly by poly(I:C) 18 h before infection. In contrast to stimulation of both subsets of BM-DCs, stimulation of L929 cells by poly(I:C) did not induce significant expression of IFNβ mRNA (Fig. 8A). Moreover, the direct antiviral effect of poly(I:C) on L929 could only be partially prevented by the IFNβ neutralizing antibodies (Fig. 8B), suggesting that the antiviral effects of poly(I:C) on these cells are probably mediated by additional IFNs or other antiviral compounds.

4. Discussion

Although it is well recognized that certain TLR ligands are able to stimulate the release of type I IFN e.g. by DC, their real antiviral potential has been studied less extensively. In this study we demonstrated that stimulation of both pDC as cDC with different TLR ligands (in particular CpG and Poly(I:C)) indeed inhibits viral infection. We also demonstrated that in this setting not IFNα, but IFNβ is the most important type I IFN, although we cannot exclude that other IFNs contribute to the TLR-mediated antiviral effects. Interestingly, the TLR3 ligand poly(I:C) showed the most pronounced antiviral effect, which was even maintained in the absence of DCs.

Although both DC subtypes are involved in the antiviral response, it has been shown that primarily the stimulation of the pDC via either TLR7 or TLR9 induces the release of massive amounts of type I IFNs, which is crucial for the initiation of an adequate immune response (Cao and Liu, 2007; Ito et al., 2005; Liu, 2005). Whether this indeed results in an adequate antiviral response has been studied less extensively. Here we demonstrate that stimulation of the FL BM-DC culture with the TLR9 ligand CpG produces an antiviral "cocktail" which markedly inhibited HSV-1 infection of L929 cells. The high mRNA expression of TLR9 (Supplemental Fig. S1a) in the FL BM-DCs strengthens the observation that the CpG-dependent antiviral effect is mediated through stimulation of TLR9. Although TLR9 mRNA could also be detected

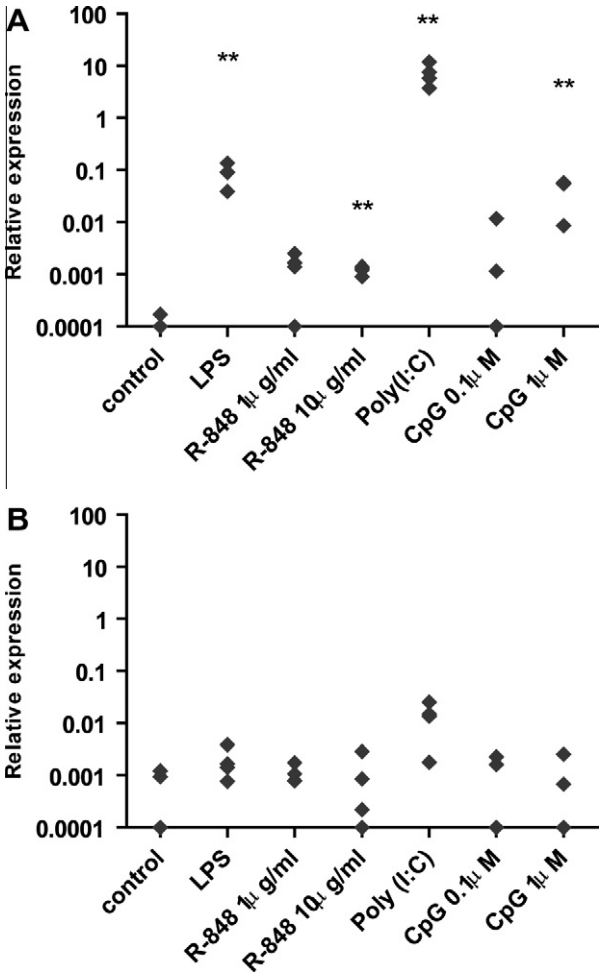

Fig. 5. Differential IFNβ expression in both BM-DC subsets. IFNβ expression in FL BM-DCs (A) and GM BM-DCs (B). IFN is displayed as relative expression compared to GAPDH values. Symbols indicate BM-DCs from individual mice ($n = 4$). $^{**}P < 0.01$ and $^{*}P < 0.05$ versus control.

in GM BM-DCs, CpG stimulation of this cell population seems inadequate to inhibit HSV-1 infection. These results are in line with previous data demonstrating that in particular pDCs respond to CpG via TLR9 resulting in the release of high amounts of type I IFN (Guiducci et al., 2006; Krug et al., 2001; Lore et al., 2003).

Although earlier studies demonstrated immuno-modulating effects and IFNα production by R848-stimulated pDCs (Gibson et al., 2002; Hemmi et al., 2002; Lore et al., 2003), we did not observe significant antiviral effect when FL BM-DCs were stimulated with the TLR7 agonist R848. Also, no or only little evidence was found for the production of type I IFN despite the fact that significant amounts of TLR7 mRNA could be detected in the cell cultures (Supplemental Fig. S1B). The reason for this discrepancy is not entirely clear, but could be due to the fact that others have used pDC-enriched cell populations (Gibson et al., 2002; Hemmi et al., 2002; Lore et al., 2003), while differentiation of murine bone marrow cells with Flt-3L results in a mixed population of predominantly pDCs, but also cDCs (Brawand et al., 2002; Gilliet et al., 2002; Yasuda et al., 2009). Also, there might be a species difference as most studies demonstrating the release of massive amounts of type I IFN have used human pDCs. Furthermore, our data are in line with Kim et al. who used a similar method to differentiate immature murine BM cells and also found little evidence for type I IFN production by R848-stimulated FL BM-DC (Kim et al., 2007). Overall, these data suggest that, at least in mice, the antiviral potency of TLR7 agonists seems limited.

Protective effects of intranasal LPS administration have previously been shown in a mouse model of HSV-1 encephalitis (Boivin et al., 2008). Here, we also demonstrate a significant antiviral effect of supernatants from both LPS-stimulated FL and GM BM-DC cultures, which corresponds with the presence of TLR4 mRNA in both cell cultures (Supplemental Fig. S1C). However, this protective effect was limited compared to the antiviral effect of CpG stimulation. Despite this observed antiviral effect of LPS, in vivo LPS treatment usually results in the release of pro-inflammatory cytokines. Therefore, despite the observed antiviral effects the therapeutic use of this TLR ligand as an antiviral drug will be limited.

Regarding the TLR3 ligand poly(I:C), the supernatants of both poly(I:C)-stimulated FL and GM BM-DC cultures impressively limited HSV-1 infection demonstrating that this dsRNA mimic is clearly a strong inducer of antiviral responses. Interestingly, poly(I:C) also strongly inhibited HSV-1 infection when directly added to L929 cells. However, in L929 cells the mRNA expression of TLR3, the receptor for poly(I:C), is very low compared to BM-DCs (Supplemental Fig. 1SD). This indicates that the stimulation of L929 cells by poly(I:C) is probably not exclusively a TLR3-mediated event but may rely on activation of cytoplasmic receptors. Indeed, melanoma-differentiation-associated gene 5 (MDA5) has been shown to be a receptor for poly(I:C) and is present in most cells (Gitlin et al., 2006; Kato et al., 2006; Kawai and Akira, 2010; Wilkins and Gale, 2010). Additional experiments have to confirm whether MDA5 acts as a receptor for poly(I:C) in L929 cells, but our results show that poly(I:C) might be a promising future antiviral drug. This is supported by recent data showing that prophylactic treatment with poly(I:C) provides a high level of protection against challenge with different viruses (Boivin et al., 2008; Gill et al., 2006; Wong et al., 2009).

Surprisingly, the TLR-mediated antiviral effects were basically IFNα independent. Usually this IFN subtype is one of the first IFNs produced following a viral infection (Baig and Fish, 2008; Katze et al., 2002). However, we were unable to detect significant amounts IFNα4 mRNA in any of the cell cultures and this observation was supported by neutralization assays using an IFNα antibody. In contrast, we found high levels of both IFNβ mRNA and protein in TLR-stimulated FL BM-DC cell cultures while the antiviral effects of these TLR ligands were almost completely abolished with an IFNβ neutralizing antibody. This strongly suggests that the antiviral effect induced by stimulation of FL BM-DCs with TLR ligands largely depends on the production of IFNβ.

Alternatively, recent data demonstrated that GM BM-DCs are less able to produce IFNβ (Schmitz et al., 2007) in response to various TLR ligands. Also in our hands TLR stimulation did not markedly enhance the expression of IFNβ mRNA in GM BM-DCs. Moreover, the antiviral effect of TLR-stimulated GM BM-DC supernatants could only partially be blocked by the IFNβ antibody, suggesting that other pathways also contribute significantly to the observed antiviral effects. Interestingly, Lauterbach et al. (2010) recently demonstrated that cDC in response to poly(I:C) release large amounts of IFN-λ a type III IFN), a recently identified new member of IFN family with potent antiviral effects against a variety of viruses including HSV-1 (Li et al., 2011).

Also the DC-independent antiviral effects of poly(I:C) seem to rely only partially on IFNβ. Stimulation of L929 cells with poly(I:C) only marginally increased the expression of IFNβ mRNA and the neutralization assay confirmed that the protective effect was indeed not entirely due to IFNβ. Nevertheless, a strong antiviral effect was observed when L929 cells were stimulated with this TLR ligand. Future experiments are mandatory to further unravel whether type III IFNs also contribute to this DC-independent effect of poly(I:C) or whether other molecular pathways are involved.

In conclusion, in this study we have demonstrated that certain TLR ligands (e.g. CpG) do have a strong DC-dependent antiviral

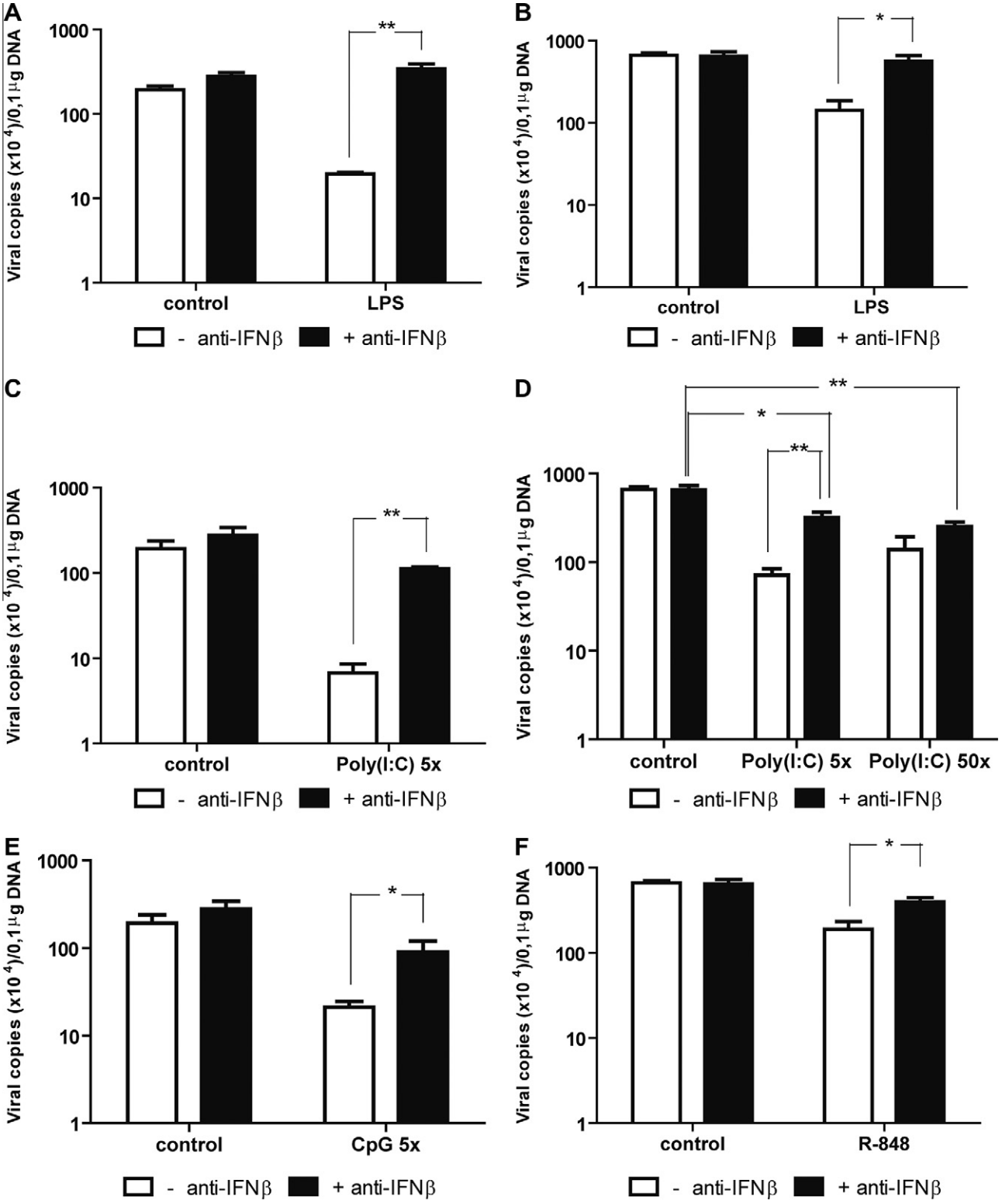

Fig. 6. IFNβ is essential for inhibition of viral replication. IFNβ neutralization of supernatant from FL BM-DCs (A,C,E) and GM BM-DCs (B,D,F). 5× or 50× indicates dilution factor of supernatant. Data represent the mean +/− SEM of three or four independent experiments. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus control.

capacity. In particular the presence of pDCs strongly enhances the antiviral effects of the TLR ligands. Interestingly, we found that IFNα seems dispensable for the TLR-induced antiviral effects and that IFNβ might be more important that IFNα. Moreover, we demonstrated a strong antiviral effect of the dsRNA mimic poly(I:C), which did not require the presence of DCs and may involve other IFNs than type I IFNs. In particular type III IFNs, which have recently been shown to have a prominent role against e.g. respiratory infections, might be engaged. This opens attractive opportunities for the use of such dsRNA mimics as prophylactic

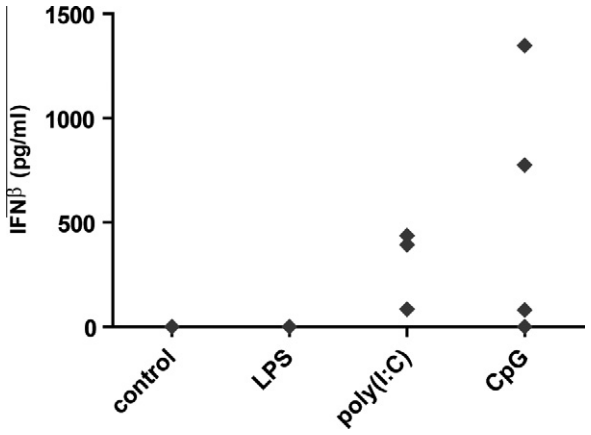

Fig. 7. Variable IFNβ production in supernatant of TLR-stimulated FL BM-DCs. IFNβ was measured in the supernatant from stimulated BM-DCs by ELISA. Symbols indicate FL BM-DC supernatant from individual mice ($n = 4$).

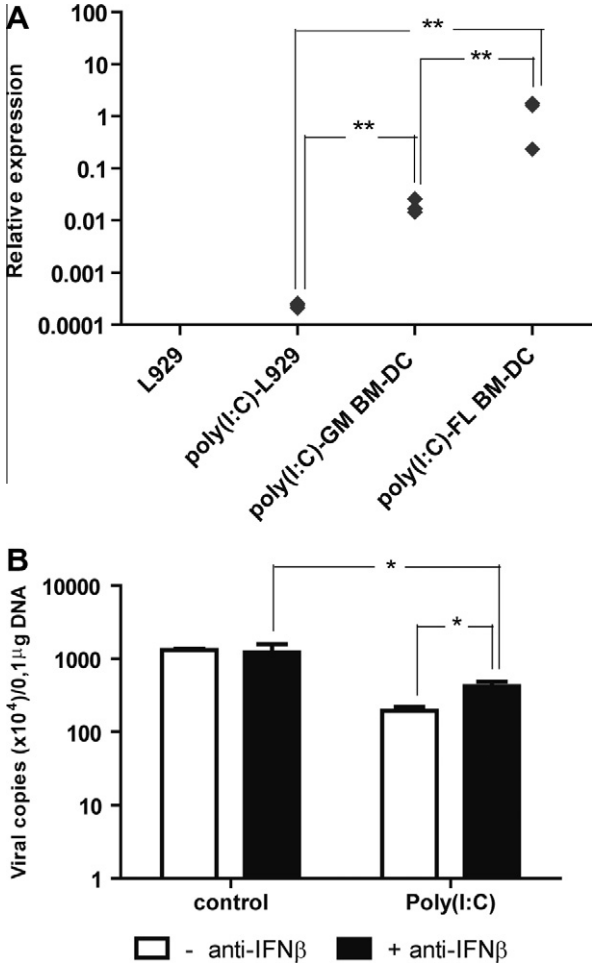

Fig. 8. Limited role for IFNβ in poly(I:C) stimulated L929 cells. (A) IFNβ expression in L929 cells after poly(I:C) stimulation compared to expression in poly(I:C)-stimulated BM-DCs IFN is displayed as relative expression compared to GAPDH values. Symbols indicate individual experiments with L929 cells or BM-DCs from individual mice ($n = 3$). (B) IFNβ neutralization from supernatant of poly(I:C) stimulated L929 cells. Data represent the mean +/− SEM of three independent experiments. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus control.

antiviral therapies e.g. for COPD patients who suffer frequently from acute exacerbations related to respiratory viral infections.

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Conflict of interest statement

The authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2011.10.015.

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