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Abstract: We have found out that transfection of the RTG-2 cell line with the viral haemorrhagic septicaemia virus (VHSV) glycoprotein G (GVHSV)-coding plasmid induces an anti-VHSV state, similar to that induced by poly I:C. Taking the advantage of the constitutive expression of toll-like receptor 9 gene (tlr9) in RTG-2 cells, we have investigated whether this antiviral state was induced by the cytosine-phosphodiester-guanine (CpG) motifs present in the plasmid DNA, by the endogenous expression of GVHSV protein or by both elements For that, we have analysed the expression profile of the rainbow trout tlr9 and several genes related to TLR9-mediated immune response in the absence or presence of a lysosomotropic drug that specifically blocks TLR9-CpG DNA interaction. The results suggested that the high levels of cell protection conferred by a plasmid encoding GVHSV gene are due to GVHSV rather than to the CpG motifs within plasmid DNA. Therefore, plasmid DNA might not play a key role in the immune response elicited by DNA vaccines or perhaps other receptors instead TLR9 could be implicated in CpG motifs recognition and signalling. In addition, since RTG-2 cells express tlr9 gene, this cell line could be a good tool for screening TLR9 agonists, such as the immunomodulatory oligonucleotides (IMOs), as fish DNA vaccine adjuvants.

Instituto de Biología Molecular y Celular
UNIVERSIDAD MIGUEL HERNÁNDEZ

Vaccine Editorial Office

Elche, November 5, 2010

Dear Sir,

Please find enclosed the revised version of the manuscript "*In vitro* analysis of the factors contributing to the antiviral state induced by a plasmid encoding the viral haemorrhagic septicaemia virus glycoprotein G in transfected trout cells " by M. Ortega-Villaizan et al. We hope this new version will be ready for publication.

Thank you very much in advance. Best wishes,

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Response to reviewer/editor

Thank you very much for your valuable comments. We have included in the new version of the manuscript the advices of reviewer 4. We hope this new version will be ready for publication.

Sincerely,

Prof. A Estepa

REVIEWER 4:

Comments:

1.- Statistical analyses are generally accepted to be optimal.

We agree, so statistical analyses have been included in the new version of the manuscript.

2.- It is generally preferable to describe sample size and replication strategy in the text.

Reviewer is right, we have included it in the text, in the material and methods section 2.7, page 9.

ABSTRACT

We have found out that transfection of the RTG-2 cell line with the viral haemorrhagic septicaemia virus (VHSV) glycoprotein G (G_{VHSV})-coding plasmid induces an anti-VHSV state, similar to that induced by poly I:C. Taking the advantage of the constitutive expression of toll-like receptor 9 gene (*tlr9*) in RTG-2 cells, we have investigated whether this antiviral state was induced by the cytosine-phosphodiester-guanine (CpG) motifs present in the plasmid DNA, by the endogenous expression of G_{VHSV} protein or by both elements For that, we have analysed the expression profile of the rainbow trout *tlr9* and several genes related to TLR9-mediated immune response in the absence or presence of a lysosomotropic drug that specifically blocks TLR9-CpG DNA interaction. The results suggested that the high levels of cell protection conferred by a plasmid encoding G_{VHSV} gene are due to G_{VHSV} rather than to the CpG motifs within plasmid DNA. Therefore, plasmid DNA might not play a key role in the immune response elicited by DNA vaccines or perhaps other receptors instead TLR9 could be implicated in CpG motifs recognition and signalling. In addition, since RTG-2 cells express *tlr9* gene, this cell line could be a good tool for screening TLR9 agonists, such as the immunomodulatory oligonucleotides (IMOs), as fish DNA vaccine adjuvants.

In vitro analysis of the factors contributing to the antiviral state induced by a plasmid encoding the viral haemorrhagic septicaemia virus glycoprotein G in transfected trout cells

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Keywords: Innate immunity; Toll-like receptor; TLR9; DNA vaccine; Viral glycoproteins; VHSV; rhabdovirus; rainbow trout; RTG-2 cell line; TNFα; IL1ß; IRF3; IRF7;Mx;unmethylated CpG motifs

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ABSTRACT

We have found out that transfection of the RTG-2 cell line with the viral haemorrhagic septicaemia virus (VHSV) glycoprotein G (G_{VHSV})-coding plasmid induces an anti-VHSV state, similar to that induced by poly I:C. Taking the advantage of the constitutive expression of toll-like receptor 9 gene (*tlr9*) in RTG-2 cells, we have investigated whether this antiviral state was induced by the cytosine-phosphodiester-guanine (CpG) motifs present in the plasmid DNA, by the endogenous expression of G_{VHSV} protein or by both elements For that, we have analysed the expression profile of the rainbow trout *tlr9* and several genes related to TLR9-mediated immune response in the absence or presence of a lysosomotropic drug that specifically blocks TLR9-CpG DNA interaction. The results suggested that the high levels of cell protection conferred by a plasmid encoding G_{VHSV} gene are due to G_{VHSV} rather than to the CpG motifs within plasmid DNA. Therefore, plasmid DNA might not play a key role in the immune response elicited by DNA vaccines or perhaps other receptors instead TLR9 could be implicated in CpG motifs recognition and signalling. In addition, since RTG-2 cells express *tlr9* gene, this cell line could be a good tool for screening TLR9 agonists, such as the immunomodulatory oligonucleotides (IMOs), as fish DNA vaccine adjuvants.

1. Introduction

A direct relationship between the viral haemorrhagic septicaemia virus (VHSV) glycoprotein G (G_{VHSV}) expression and type I interferon (IFN) production has been described in the rainbow trout cell line RTG-P1 [1]. However, there are no records on cell resistance to VHSV infection induced by cell transfection with a G_{VHSV}-coding plasmid. Regarding this issue, among the factors contributing to this *in vitro* potential cell protection are the antigenic properties of G_{VHSV} protein as well as the unmethylated cytosine-phosphodiestesine (CpG) motifs within the bacterial DNA plasmid.

It is known that bacterial DNA is immunogenic in the vertebrate host [2-4] because of the presence of CpG motifs that activate the innate immune system by means of toll like receptor 9 (TLR9) [5-7], a member of the pattern recognition receptor (PRR) family. The TLR9-mediated recognition of bacterial DNA and CpG-containing oligodeoxynucleotides (CpG-ODNs) induces cell production of type I interferon (IFN) and pro-inflammatory cytokines.

To date, the majority of studies about the immune effect of unmethylated CpG motifs have been carried out on mammals where they are proving to be very successful at stimulating innate and adaptive immune responses in a variety of species as well as protecting them from bacterial, viral and protozoan pathogens [4, 8, 9]. However, more effort is now being focused on evaluating, both *in vitro* and *in vivo*, the immunostimulatory effects of those pathogen associated molecular patterns (PAMPs) in veterinary-relevant species including cultured teleost fish[4, 10, 11].

Concerning cultured fish, it is necessary to point out that aquaculture has become an important industry in the supply of fish of sufficient quality and quantity for human consumption to replace wild-fishing. However, factors such

as viral infectious diseases continue to remain an impediment to the development, productivity and profitability of fish farms. Consequently, all the approaches conducted to enhance the fish immune defences, including the search for DNA vaccines adjuvants, are of definitive interest.

The innate immune recognition of the adjuvant elements of vaccine formulations have been shown to be critical for its immunogenicity [12, 13]. In the case of DNA vaccines, CpG motifs within plasmid DNA have been considered to be 'built-in' adjuvants, owing to their apparent ability to activate the innate immune system by means of TLR9 [14-18]. However, whether the immunogenic properties of plasmid DNA involve TLR9 signalling or not has not been clearly established [13, 19, 20].

Taking into account the increasing interest to establish whether the immunogenic properties of plasmid DNA involve TLR9 signalling, in this work we have analysed the contribution of both G_{VHSV} protein and plasmid DNA backbone CpG motifs to the *in vitro* cell protection against VHSV infection. For that, the RTG-2 cell line was chosen because of the constitutive expression of the *tlr9* gene, not previously described in this cell type.

The results have shown that high levels of cell resistance to VHSV infection, associated with high expression levels of an interferon-induced antiviral gene *mx,* tumour necrosis factor alpha (*tnf*α) and interleukin 1 beta (*il1*β) genes were only achieved in RTG-2 cells transfected with a G_{VHSV}–coding plasmid.

On the other hand, inhibition of endosomal acidification using lysosomotropic drugs that specifically blocks TLR9-CpG DNA interaction [21] almost left unchanged the response induced by unmethylated CpG ODNs or plasmid DNA but highly down-regulated the response induced by G_{VHSV}. However,

the down-regulation of G_{VHSV}-induced immune-related response directly correlated with the decrease of the *G_{VHSV}* gene transcription.

All together, our results suggest that the high levels of cell protection conferred by a plasmid encoding G_{VHSV} gene seem to be due to G_{VHSV} rather than to the CpG motifs within plasmid DNA. Therefore, plasmid DNA might not play a key role in the immune response elicited by DNA vaccines or instead other receptors rather than TLR9 could be implicated in CpG motifs recognition and signalling. In addition, since RTG-2 cells express *tlr9* gene this cell line could be a good tool for screening TLR9 agonists, such as the immunomodulatory oligonucleotides (IMOs) [22], as fish DNA vaccine adjuvants.

2. Material and Methods

2.1. Cell cultures and virus

Rainbow trout cell line RTG-2 (rainbow trout gonad-2) purchased from the American Type Culture Collection (ATCC CCL 55) was used in this work. RTG-2 cells were maintained at 20 °C in a 5 % CO_2 atmosphere with MEM cell culture medium (Sigma, St. Louis, MO) containing 10 % Fetal Bovine Serum (FBS) gamma irradiated (Cultek, Madrid, Spain), 2 mM L-glutamine (Gibco, Invitrogen Corporation, United Kingdom) and 50 µg/mL gentamicin (Gibco).

Viral haemorrhagic septicaemia virus (VHSV-07.71) [23] isolated in France from rainbow trout, *Oncorhynchus mykiss*, was propagated in EPC cells at 14 ºC as previously reported [24]. Supernatants from VHSV-07.71 infected EPC cell monolayers were clarified by centrifugation at 1.000 x g during 20 min and kept in aliquots at –80 ºC. Clarified supernatants were used for the experiments. The virus stock was titrated in 96-well plates using a previously developed immunostaining focus assay (focus forming units, f.f.u.) [25-27].

2.2. Transfection assays

RTG-2 cells were transfected with the pAE6 plasmid [28, 29] or with the plasmid encoding the VHSV glycoprotein G (G_{VHSV}) gene (pAE6-G) [30]. Cell transfections were carried out as described previously [29, 31, 32]. Briefly, RTG-2 cells, grown in culture flasks of 75 cm^2, were detached using TrypLE™ Select (Gibco), washed, resuspended in MEM supplemented with 10 % FBS and dispensed into 96-well plates at a concentration of $4x10^4$ cells per well in a final volume of 100 µl. Then, 0.25 µg of pAE6 or pAE6-G plasmids complexed with 0.3 µl of FuGene HD (Roche Diagnostics, Mannheim, Germany) were added to each

well and the plates were further incubated at 20 ºC for 3 days. After the incubation period, the medium was removed and total RNA extracted as described in 2.6.

2.3. Treatment of RTG-2 cells with unmethylated CpG ODNs and poly I:C

RTG-2 cells, grown in 96–well plates, were incubated 24 hours at 20 ºC with each of the synthetic CpG ODNs (6 µM), 30 µg/ml of Polyribocytidylic acid (poly I:C) (Sigma) or left untreated. After the incubation period, the medium was removed and total RNA extracted as described in 2.6.

The synthetic unmethylated CpG ODNs used in the present work were: CpG ODN 2133 (T**CG**T**CG**TTGGTTGT**CG**TTTTGGT), CpG ODN 2143 (TT**CG**T**CG**TTTTGT**CG**TTTTGT**CG**TT), and non-CpG ODN 1982 (TCCAGGACTTCTCTCAGGTT), purchased from Sigma, and resuspended in sterile water at 1 mM.

2.4. Infection of RTG-2 cells with VHSV

RTG-2 cells, grown in 96-well plates, were treated with CpG ODNs (2133, 2143), the non-CpG ODN 1982, or poly I:C during 24 hours at 20 ºC or transfected with pAE6 or pAE6-G during 72 hours at 20 ºC. Then, cell monolayers were washed extensively with PBS and then infected with VHSV at a multiplicity of infection (m.o.i.) of 10^{-2}, in a final volume of 100 µl/well of cell culture medium supplemented with 2 % FBS. After 2 hours at 14 °C, infected RTG-2 cells were extensively washed with PBS, 100 µl/well of cell culture medium supplemented with 2 % FBS added to each well and plates further incubated at 14 °C for 22 hours. After incubation period, cells were washed

extensively with PBS, total RNA extracted and VHSV replication evaluated by real-time PCR as described in 2.6 and 2.7, respectively, using specific primers and probe for the gene encoding the protein N of VHSV (N_{VHSV}) (Table 1). Untreated RTG-2 cells infected with VHSV were included as controls.

2.5. Blocking of endosome acidification by NH_4Cl

To block endosomal low-pH NH_4Cl (Sigma) at 10 mM was added to RTG-2 cell monolayers during 1 hour. Cell monolayers were then treated with CpG ODNs (2133, 2143), non-CpG ODN 1982, poly I:C during 24 hours at 20 ºC or transfected with pAE6 or pAE6-G during 72 hours at 20 ºC, in the presence of NH_4Cl. No significant cell death was observed in RTG-2 cell monolayers treated with NH_4Cl 10mM, since this concentration is known as non-cytotoxic but effective for reducing VHSV infectivity by 50 % [26]. After the incubation period, the medium was removed and total RNA extracted as described in 2.6.

In addition, we carried out a time course of low-pH cell environment recovery after blocking endosomal acidification with NH_4Cl. For that, RTG-2 cells were pretreated with 10 mM NH_4Cl and then transfected with pAE6-G. NH_4Cl was retired at the following times: before transfection, a quarter of hour, half and hour, 2, 3, 4, 6 and 7 hours after transfection. After removing the NH_4Cl, fresh medium was added and plates further incubated until 24 hours (for CpG ODNs and poly I:C treatments) or 72 hours (for transfected cells). Finally, the medium was removed and total RNA extracted as described in 2.6.

2.6. RNA isolation and cDNA synthesis

Total RNA was extracted from the treated, transfected and/or infected RTG-2 cells using the "RNeasy® Plus Mini" kit (Qiagen, Valencia, CA), following

manufacturer's instructions. Isolated RNAs were resuspended in DNase and RNase free water (Sigma) and stored at -80 °C until used. Two hundred ng of total RNA were used for cDNA synthesis using the Moloney murine leukaemia virus reverse transcriptase (M-MLV) (Invitrogen) and Random Hexamers (Applied Biosystems, NY), following manufacture's instructions.

2.7. Gene expression analysis

Real-time PCR assays were performed using the ABI PRISM® 7300 Sequence Detector System (Applied Biosystems). Reactions were carried out in a final volume of 20 µl, containing 900 nM of each primer, 200 nM of the TaqMan® probe conjugated with the fluorescein FAM at the 5' end and with the quencher TAMRA at the 3', 20 ng of RNA and 1× TaqMan® Universal Master Mix (Applied Biosystems). Primers and probes sequences are indicated in Table 1. For *il1ß* and *irf7* gene primers SYBR Green (Applied Biosystems) was used. Primers for *irf7* and *mx* genes amplification were designed using the Primer Express™ software (Applied Biosystems). *mx* gene primers amplify isoforms one and three, since they are the Mx protein isoforms predominantly expressed in RTG-2 cells in response to different type I IFN inducers [31]. Thermal cycling conditions followed the standard default protocol of the instrument. Gene expression results were analyzed using the $2^{-\Delta\Delta Ct}$ method [33]. Endogenous control for quantification was the *ef1*α gene [34]. Data represent the mean fold changes ± SD for three independent experiments, each performed in duplicate.

For normal PCR assays, PCR amplification reactions (25 µl) were performed using 0.5 µl dNTP mix (10mM each), 0.2 µl Taq DNA polymerase (Roche, Barcelona, Spain), 2.5 µl Taq 10× buffer, 0.5 µl of each primer (20 µM) (Table 1) and 2.5 µl of cDNA. A parallel PCR with primers for glyceraldehyde 3-

phosphate dehydrogenase (GAPDH) [35] (Table 1) was performed with all samples as a house-keeping gene using conditions previously described [31, 36]. PCRs were carried out in a GeneAmp® PCR System 2700 cycler (Applied Biosystems). PCR products (10 µl) were visualized on a 2 % agarose gel stained with ethidium bromide.

2.8. Statistical analysis

Data were analyzed using an analysis of variance (ANOVA) and a Tukey's multiple comparison test to determine the differences between the different treatments and control cells.

2.9. Rainbow trout Mx protein detection by immunofluorescence

RTG-2 cells, grown in 96–well plates, were incubated with each of the synthetic CpG ODNs (6 µM), 30 µg/ml of poly I:C (Sigma), or transfected with 0.25 µg/well of pAE6 and pAE6-G as indicated above, during 72 hours at 20 ºC. In addition, untreated RTG-2 cells were included as controls. After the incubation period, the cell culture medium was removed, and the cell monolayers were fixed with BD Cytofix (BD Biosciences, Franklin Lakes, NJ) (15 min. at room temperature) and permeabilized 5 min. at room temperature with 0.2 % Triton X100 (Merck, Darmstadt, Germany). Then, cell monolayers were incubated with the antiserum to rainbow trout Mx protein [37], diluted 300-fold in PBS-0.1 % BSA, during 2:30 hours at room temperature. This antibody recognizing the three isoforms of the rainbow trout Mx protein was detected by using a goat anti-rabbit antibody TRITC conjugated (Sigma). Stained cells were viewed and photographed with an inverted fluorescence microscope (Nikon Eclipse TE2000-U, Nikon instruments Inc., NY) provided with a digital camera (Nikon DS-1QM).

3. Results

3.1. VHSV infectivity in treated or transfected RTG-2 cells

Transfection of the RTG-2 cell line with pAE6-G induced similar protection against VHSV infection (VHSV infectivity ≤6 %) than that induced by poly I:C treatment (Figure 1). RTG-2 cells treated with non-CpG ODN 1982 or transfected with pAE6 propagated the virus almost as efficiently as the untreated cells (Figure 1). It is worth noting that pAE6 contents three CpG rich regions as calculated by the software EMBOSSCpGPlot (http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html) (data not shown). On the other hand, RTG-2 cells treated with CpG ODNs, mainly with CpG 2133, showed some resistance to VHSV infection (VHSV infectivity ~ 70%) (Figure 1).

3.2. Expression profile of rainbow trout tlr9 gene and other genes related to TLR9-mediated immune response in treated or transfected RTG-2 cells

As a first step, normal PCR was performed using total RNA extracted from untreated or transfected RTG-2 cells to investigate the expression of rainbow trout *tlr9* gene in this cell line. *tlr9* gene transcription was detectable in untreated cells as well as in transfected cells (Figure 2A).

Having established the *tlr9* gene expression in RTG-2 cells, we analysed whether the resistance to VHSV infection correlated with *tlr9* and *tlr9*-related genes expression. No differential expression of *tlr9* gene was observed in response to pAE6, CpG ODNs, or non-CpG ODN since these cells showed similar *tlr9* basal expression levels than untreated RTG-2 cells (Figure 2B). However, an up-regulation of *tlr9* gene was detected in pAE6-G transfected cells (~1.5-fold) and poly I:C treated cells (~2-fold) (Figure 2B).

TLR9-mediated immune response was evaluated by analysing the expression of interferon regulatory factors *irf3* and *irf7*, the interferon stimulated (IS) *mx* gene and the pro-inflammatory cytokines *tnfα* and *il1ß,* in cells stimulated with the above indicated PAMPs. To evaluate type I IFN response, the IFN-inducible *mx* gene was chosen since Mx proteins have been proven to be a very specific and sensitive marker for type I IFN induction [38-40] and direct induction of this gene by rainbow trout type I IFN has been demonstrated [41].

The up-regulation of *tlr9* gene associated to high cell resistance to VHSV correlated with high expression levels of *mx* (~1600-fold)*, tnfα* (~29-fold), *il1ß* (~6-fold)*, irf3* (~16-fold) and *irf7* (~48-fold) in the RTG-2 cells transfected with pAE6-G (Figure 2B). On the other hand, minor accumulation of *mx, tnfα*, *il1ß*, *irf3* and *irf7* gene transcripts was observed after cell transfection with pAE6 or treatment with CpG ODNs (Figure 2B).

The expression of *mx* gene was also evaluated at the protein level using an antiserum to Mx protein. RTG-2 cells treated with CpG ODNs (2133, 2143), non-CpG ODN 1982, or transfected with pAE6 showed no increment on the Mx protein expression levels, compared to untreated RTG-2 cells, as demonstrated by immunofluorescence (data not shown). In contrast, RTG-2 cells treated with poly I:C (Figure 3, middle panel) or transfected with pAE6-G (Figure 3, lower panel) showed high expression levels of Mx protein.

3.3. Influence of the low-pH endosome environment in the immune response induced by CpG ODNs, pAE6 and pAE6-G

Since endosomal acidification is a prerequisite for TLR9 activation, to further examine its implication in CpG ODNs, pAE6 and pAE6-G immune-related response, we blocked endosomal acidification by using NH_4Cl.

Blocking endosomal acidification highly decreased *mx* (~100-fold), *il1ß* (~3-fold), *tnfα* (~13-fold), *irf3* (~15-fold) and *irf7* (~47-fold) gene expression in pAE6-G transfected RTG-2 cells (Figure 4). In contrast, the expression of these genes in response to CpG ODNs or pAE6 remained almost unchanged after treating the cells with NH_4Cl, except for an up-regulation of *mx* gene expression by pAE6 (~2-fold), as well as for a down-regulation of *irf7* by CpG 2133 and 2143 (~2-fold, both of them) (Figure 4). Low-pH endosome blocking failed to produce any change in the *mx* gene expression induced by poly I:C (Figure 4). Nevertheless, it resulted in a down-regulation of *il1ß* gene (~3-fold) (Figure 4).

On the other hand, *tlr9* gene expression remained unchanged upon NH_4Cl treatment, except for pAE6-G transfected cells where *tlr9* gene transcription diminished (~1,3-fold) (Figure 4).

To analyse the cause of the reduced IFN-mediated response after NH_4Cl treatment, we carried out a time course of low-pH cell environment recovery after blocking endosomal acidification with NH_4Cl. We observed that the progressive delayed retirement of NH_4Cl caused a time-dependent reduction of G_{VHSV} gene expression which coupled with the decrease of *mx* gene transcript level (Figure 5).

4. Discussion

In the present work, transfection of the RTG-2 cell line with pAE6-G induced an anti-VHSV state similar to that induced by poly I:C. After, we further proceeded to evaluate whether this protection was induced by the endogenous expression of G_{VHSV} protein or by the CpG motifs within plasmid DNA backbone.

Different immune responses such as the induction of type I IFN system and the production of TNFα and IL1ß are triggered after the vertebrate innate immune system recognise the CpG motifs present in bacterial DNA as well as in synthetic CpG ODNs [42]. TLR9-mediated recognition of CpG-containing oligodeoxynucleotides (CpG ODNs) has been shown to induce the production of type I IFN and pro-inflammatory cytokines [43]. Regarding fish, the CpG 2133 and 2143, belonging to C-class ODNs are known to enhance the expression of IL1ß in RTS11 cells (rainbow trout monocyte/macrophage-like cell line) and to activate *mx* gene promoter in transformed RTG-2 cells [4]. However, our results showed low cell resistance to VHSV and lack of immune-related response induced by these CpG ODNs, evaluated as *tlr9*, *mx*, proinflammatory cytokines (*tnfα* and *il1ß*) and *irf3* genes regulation, as well as *mx* protein expression. Only, a minor up-regulation of *irf7* was observed in response to these CpG ODNs. Minor alterations in *tlr9* gene transcripts have been observed in response to CpG ODNs in Atlantic salmon [44].

RTG-2 cells transfected with pAE6-G showed an up-regulation of rainbow trout *tlr9* gene associated to high cell resistance to VHSV, which correlated with high expression levels of *mx, tnfα*, *il1ß, irf3* and *irf7* genes, as well as with high *mx* protein expression. Although this result could initially suggest that plasmid DNA recognition by TLR9 is contributing to this immune-related response.,

Taking into account the fact that a minor accumulation of *mx, tnfα*, *il1ß*, *irf3* and *irf7* gene transcripts was observed after cell transfection with pAE6 or treatment with CpG ODNs, this assumption should be discarded. In view of these results, we suggest a possible up-regulation of the rainbow trout *tlr9* gene by means of the IFN induced by G_{VHSV}, but not by means of the CpG motifs within the plasmid DNA or in the synthetic unmethylated CpG ODNs used in this study. Similarly, modulation of *tlr9* gene expression by IFN has been previously described in several animal species including fish [45, 46].

IRF3 pathway has been shown to be responsible for TLR3/TLR4-specific gene activation, leading IRF3 to the induction of a set of primary and secondary genes (among them *irf7* and *mx*) involved in host defence [47]. Further, a direct implication of IRF7 in type I IFN production after TLR4/CD14 recognition of the vesicular stomatitis virus glycoprotein G (G_{VSV}) has been described [48]. We have observed a direct correlation between *irf3*, *irf7* and *mx* gene up-regulation and pAE6-G transfection, but not by pAE6; however, we only observed a minor up-regulation of *irf7* after CpG ODNs stimulation. These results might indicate that CpG ODNs could be triggering a weak signal through TLR9; however, other TLRs should be triggering IFN and proinflammatory cytokines production after pAE6-G transfection through IRF3 primarily and IRF7 secondarily.

Once activated TLR9 is transported to late endosome/lysosomes [49]. Then, TLR9-CpG-DNA interaction occurs at the acidic pH found in endosomes and lysosomes [21]. In macrophages, late endosome/lysosome-localized Rab7b, a small GTPase that plays a role in the transport and degradation of proteins in endosomes and lysosomes in mammalian cells, is known to down-regulate TLR9-triggered proinflammatory cytokines and type I IFN production by impairing TLR9 signalling via promotion of TLR9 degradation [49]. In a similar way, our results

showed that blocking endosomal acidification using NH_4Cl highly decreased *mx*, *il1ß*, *tnfα*, *irf3* and *irf7* genes transcription in the RTG-2 cells transfected with pAE6-G. Therefore, cell treatment with NH_4Cl caused a drastic reduction in the genes previously up-regulated by pAE6-G transfection, consistent with a role for TLR9 activation in cytokine gene induction. However, a time course of low-pH cell environment recovery caused a time-dependent reduction of G_{VHSV} gene expression which coupled with a decrease of *mx* gene transcript level, suggesting low-pH endosome dependence for cell plasmid uptake, intracellular traffic and/or expression. Therefore, the reduction of G_{VHSV}-mediated IFN and pro-inflammatory cytokine induction by NH_4Cl is directly related to G_{VHSV} expression levels and seems to be independent of rainbow trout TLR9 activation.

All together, our results suggest that the anti-VHSV state induced by pAE6-G seem to be due to G_{VHSV} rather than to the CpG motifs within plasmid DNA and therefore plasmid DNA might not play a key role as a 'built-in' adjuvant of fish DNA vaccines, or other receptors instead could be implicated in CpG motifs recognition and signalling. Further work is in progress where an engineered CpG-modified plasmid DNA is being tested. Separately, it is worth noting that the RTG-2 fibroblastic cell line could also reveal an absence of responsiveness to CpG motifs, since other fibroblast cell lines like cultured synovial fibroblasts remained TLR9 mRNA at baseline levels after stimulation with CpG ODNs [50]. However, since RTG-2 cells express *tlr9* gene, this cell line could be a good tool for screening other TLR9 agonists, such as the immunomodulatory oligonucleotides (IMOs) [22], as fish DNA vaccine adjuvants.

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REFERENCES

[1] Acosta F, Collet B, Lorenzen N, Ellis AE. Expression of the glycoprotein of viral haemorrhagic septicaemia virus (VHSV) on the surface of the fish cell line RTG-P1 induces type 1 interferon expression in neighbouring cells. Fish Shellfish Immunol 2006 Sep;21(3):272-8.

[2] Messina JP, Gilkeson GS, Pisetsky DS. Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA. J Immunol 1991 Sep 15;147(6):1759-64.

[3] Carrington AC, Collet B, Holland JW, Secombes CJ. CpG oligodeoxynucleotides stimulate immune cell proliferation but not specific antibody production in rainbow trout (Oncorhynchus mykiss). Vet Immunol Immunopathol 2004 Oct;101(3-4):211-22.

[4] Carrington AC, Secombes CJ. A review of CpGs and their relevance to aquaculture. Vet Immunol Immunopathol 2006 Aug 15;112(3-4):87-101.

[5] Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000 Dec 7;408(6813):740-5.

[6] Medzhitov R JCJ. Innate immune recognition: mechanisms and pathways. Immunol Rev 2000;173:89-99.

[7] Bauer S, Kirschning CJ, Hacker H, Redecke V, Hausmann S, Akira S, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci U S A 2001 Jul 31;98(16):9237-42.

[8] Krieg AM. CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol 2002;20:709-60.

[9] Mutwiri GK, Nichani AK, Babiuk S, Babiuk LA. Strategies for enhancing the immunostimulatory effects of CpG oligodeoxynucleotides. J Control Release 2004 May 31;97(1):1-17.

[10] Cuesta A, Salinas I, Esteban MA, Meseguer J. Unmethylated CpG motifs mimicking bacterial DNA triggers the local and systemic innate immune parameters and expression of immune-relevant genes in gilthead seabream. Fish Shellfish Immunol 2008 Nov;25(5):617-24.

[11] Cuesta A, Esteban MA, Meseguer J. The expression profile of TLR9 mRNA and CpG ODNs immunostimulatory actions in the teleost gilthead seabream points to a major role of lymphocytes. Cell Mol Life Sci 2008 Jul;65(13):2091-104.

[12] Pulendran B, Ahmed R. Translating innate immunity into immunological memory: implications for vaccine development. Cell 2006 Feb 24;124(4):849-63.

[13] Ishii KJ, Kawagoe T, Koyama S, Matsui K, Kumar H, Kawai T, et al. TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. Nature 2008 Feb 7;451(7179):725-9.

[14] Krieg AM, Davis HL. Enhancing vaccines with immune stimulatory CpG DNA. Curr Opin Mol Ther 2001 Feb;3(1):15-24.

[15] Kurath G. Overview of recent DNA vaccine development for fish. Dev Biol (Basel) 2005;121:201-13.

[16] McCluskie MJ, Krieg AM. Enhancement of infectious disease vaccines through TLR9-dependent recognition of CpG DNA. Curr Top Microbiol Immunol 2006;311:155-78.

[17] van Duin D, Medzhitov R, Shaw AC. Triggering TLR signaling in vaccination. Trends in Immunology 2006;27(1):49-55.

[18] Tonheim TC, Bogwald J, Dalmo RA. What happens to the DNA vaccine in fish? A review of current knowledge. Fish Shellfish Immunol 2008 Jul;25(1-2):1-18.

[19] Babiuk S, Mookherjee N, Pontarollo R, Griebel P, van Drunen Littel-van den Hurk S, Hecker R, et al. TLR9-/- and TLR9+/+ mice display similar immune responses to a DNA vaccine. Immunology 2004 Sep;113(1):114-20.

[20] Ortega-Villaizan M, Chico V, Falco A, Perez L, Coll JM, Estepa A. The rainbow trout TLR9 gene and its role in the immune responses elicited by a plasmid encoding the glycoprotein G of the viral haemorrhagic septicaemia rhabdovirus (VHSV). Mol Immunol 2009 May;46(8-9):1710-7.

[21] Rutz M, Metzger J, Gellert T, Luppa P, Lipford GB, Wagner H, et al. Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner. Eur J Immunol 2004 Sep;34(9):2541-50.

[22] Kandimalla ER, Bhagat L, Li Y, Yu D, Wang D, Cong YP, et al. Immunomodulatory oligonucleotides containing a cytosine-phosphate-2'-deoxy-7-deazaguanosine motif as potent toll-like receptor 9 agonists. Proc Natl Acad Sci U S A 2005 May 10;102(19):6925-30.

[23] LeBerre M, De Kinkelin P, Metzger A. Identification sérologique des rhabdovirus des salmonidés. Bulletin Office International Epizooties 1977;87:391-3.

[24] Basurco B, Coll JM. Spanish isolates and reference strains of viral haemorrhagic septicaemia virus shown similar protein size patterns. Bulletin European Association Fish Pathology 1989;9:92-5.

[25] Lorenzo G, Estepa A, Coll JM. Fast neutralization/immunoperoxidase assay for viral haemorrhagic septicaemia with anti-nucleoprotein monoclonal antibody. J Virol Methods 1996 Apr 26;58(1-2):1-6.

[26] Mas V, Perez L, Encinar JA, Pastor MT, Rocha A, Perez-Paya E, et al. Salmonid viral haemorrhagic septicaemia virus: fusion-related enhancement of virus infectivity by peptides derived from viral glycoprotein G or a combinatorial library. J Gen Virol 2002;83(Pt 11):2671-81.

[27] Falco A, Mas V, Tafalla C, Perez L, Coll JM, Estepa A. Dual antiviral activity of human alpha-defensin-1 against viral haemorrhagic septicaemia rhabdovirus (VHSV): inactivation of virus particles and induction of a type I interferon-related response. Antiviral Res 2007 Nov;76(2):111-23.

[28] Cheng C, Lu KL, Lau EL, Yang TY, Lee CY, Wu JL, et al. Growth promotion in ayu (*Plecoglossus altivelis*) by gene transfer of the rainbow trout growth hormone gene. Zoological Studies 2002;41:303-10.

[29] Brocal I, Falco A, Mas V, Rocha A, Perez L, Coll JM, et al. Stable expression of bioactive recombinant pleurocidin in a fish cell line. Appl Microbiol Biotechnol 2006 Oct;72(6):1217-28.

[30] Chico V, Ortega-Villaizan M, Falco A, Tafalla C, Perez L, Coll JM, et al. The immunogenicity of viral haemorragic septicaemia rhabdovirus (VHSV) DNA vaccines can depend on plasmid regulatory sequences. Vaccine 2009 Mar 18;27(13):1938-48.

[31] Tafalla C, Chico V, Perez L, Coll JM, Estepa A. In vitro and in vivo differential expression of rainbow trout (Oncorhynchus mykiss) Mx isoforms in response to viral haemorrhagic septicaemia virus (VHSV) G gene, poly I:C and VHSV. Fish Shellfish Immunol 2007 Jul;23(1):210-21.

[32] Falco A, Chico V, Marroqui L, Perez L, Coll JM, Estepa A. Expression and antiviral activity of a beta-defensin-like peptide identified in the rainbow trout (Oncorhynchus mykiss) EST sequences. Mol Immunol 2008 Feb;45(3):757-65.

[33] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001 Dec;25(4):402-8.

[34] Raida MK, Buchmann K. Innate immune response in rainbow trout (Oncorhynchus mykiss) against primary and secondary infections with Yersinia ruckeri O1. Dev Comp Immunol 2009;33(1):35-45.

[35] Wang T, Johnson N, Zou J, Bols N, Secombes CJ. Sequencing and expression of the second allele of the interleukin-1beta1 gene in rainbow trout (Oncorhynchus mykiss):

identification of a novel SINE in the third intron. Fish Shellfish Immunol 2004 Mar;16(3):335-58.

[36] Falco A, Brocal I, Perez L, Coll JM, Estepa A, Tafalla C. In vivo modulation of the rainbow trout (Oncorhynchus mykiss) immune response by the human alpha defensin 1, HNP1. Fish Shellfish Immunol 2008 Jan;24(1):102-12.

[37] Chico V, Martinez-Lopez A, Ortega-Villaizan M, Falco A, Perez L, Coll JM, et al. Pepscan mapping of viral hemorrhagic septicemia virus glycoprotein g major lineal determinants implicated in triggering host cell antiviral responses mediated by type I interferon. J Virol 2010;84(14):7140-50.

[38] Simon A, Fah J, Haller O, Staeheli P. Interferon-regulated Mx genes are not responsive to interleukin-1, tumor necrosis factor, and other cytokines. J Virol 1991 Feb;65(2):968-71.

[39] von Wussow P, Jakschies D, Hochkeppel HK, Fibich C, Penner L, Deicher H. The human intracellular Mx-homologous protein is specifically induced by type I interferons. Eur J Immunol 1990 Sep;20(9):2015-9.

[40] Halminen M, Ilonen J, Julkunen I, Ruuskanen O, Simell O, Makela MJ. Expression of MxA protein in blood lymphocytes discriminates between viral and bacterial infections in febrile children. Pediatr Res 1997 May;41(5):647-50.

[41] Zou J, Tafalla C, Truckle J, Secombes CJ. Identification of a second group of type I IFNs in fish sheds light on IFN evolution in vertebrates. J Immunol 2007 Sep 15;179(6):3859-71.

[42] Klaschik S, Gursel I, Klinman DM. CpG-mediated changes in gene expression in murine spleen cells identified by microarray analysis. Mol Immunol 2007 Feb;44(6):1095-104.

[43] Kumagai Y, Takeuchi O, Akira S. TLR9 as a key receptor for the recognition of DNA. Adv Drug Deliv Rev 2008 Apr 29;60(7):795-804.

[44] Strandskog G, Skjaeveland I, Ellingsen T, Jorgensen JB. Double-stranded RNA- and CpG DNA-induced immune responses in Atlantic salmon: comparison and synergies. Vaccine 2008 Aug 26;26(36):4704-15.

[45] Schroder K, Sweet MJ, Hume DA. Signal integration between IFNgamma and TLR signalling pathways in macrophages. Immunobiology 2006;211(6-8):511-24.

[46] Skjaeveland I, Iliev DB, Zou J, Jorgensen T, Jorgensen JB. A TLR9 homolog that is up-regulated by IFN-gamma in Atlantic salmon (Salmo salar). Dev Comp Immunol 2008;32(6):603-7.

[47] Doyle S, Vaidya S, O'Connell R, Dadgostar H, Dempsey P, Wu T, et al. IRF3 mediates a TLR3/TLR4-specific antiviral gene program. Immunity 2002 Sep;17(3):251-63.

[48] Georgel P, Jiang, Z., Kunz, S., Janssen, E., Mols, J., Hoebe, K., Bahram, S., Oldstone, M. and , Bruce Beutler, B. Vesicular stomatitis virus glycoprotein G activates a specific antiviral Toll-like receptor 4-dependent pathway. Virology 2007;362(2):304-13.

[49] Yao M, Liu X, Li D, Chen T, Cai Z, Cao X. Late endosome/lysosome-localized Rab7b suppresses TLR9-initiated proinflammatory cytokine and type I IFN production in macrophages. J Immunol 2009 Aug 1;183(3):1751-8.

[50] Kyburz D, Rethage J, Seibl R, Lauener R, Gay RE, Carson DA, et al. Bacterial peptidoglycans but not CpG oligodeoxynucleotides activate synovial fibroblasts by toll-like receptor signaling. Arthritis Rheum 2003 Mar;48(3):642-50.

[51] Purcell MK, Nichols KM, Winton JR, Kurath G, Thorgaard GH, Wheeler P, et al. Comprehensive gene expression profiling following DNA vaccination of rainbow trout against infectious hematopoietic necrosis virus. Mol Immunol 2006 May;43(13):2089-106.

[52] Purcell MK, Kurath G, Garver KA, Herwig RP, Winton JR. Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection or DNA vaccination. Fish Shellfish Immunol 2004 Nov;17(5):447-62.

[53] Purcell MK, Smith KD, Hood L, Winton JR, Roach JC. Conservation of Toll-Like Receptor Signaling Pathways in Teleost Fish. Comp Biochem Physiol Part D Genomics Proteomics 2006 Mar;1(1):77-88.

[54] Chico V, Gomez N, Estepa A, Perez L. Rapid detection and quantitation of viral hemorrhagic septicemia virus in experimentally challenged rainbow trout by real-time RT-PCR. J Virol Methods 2006 Mar;132(1-2):154-9.

Table 1: Primer sequences used for gene expression analysis

Primer name	5′ – 3′ Sequence	Reference / Accesión no.
ef1α-fw	ACCCTCCTCTTGGTCGTTTC	[34]
ef1α-rv	TGATGACACCAACAGCAACA	
ef1α-probe	GCTGTGCGTGACATGAGGCA	
tlr9 fw for real-time PCR	CCTGCGACACTTCCTGGTTT	[20]
tlr9 rv for real-time PCR	GCCAGTGGTAAGAAGGAGGATCT	
tlr9 probe	CAGACTTCCTGCGTGCCGGCC	
mx fw	TGAAGCCCAGGATGAAATGG	U30253 and U47946
mx rv	TGGCAGGTCGATGAGTGTGA	
mx probe	ACCTCATCAGCCTAGAGATTGGCTCCCC	
tnfα fw	AGCATGGAAGACCGTCAACGAT	[51]
tnfα rv	ACCCTCTAAATGGATGGCTGCTT	
tnfα probe	AAAAGATACCCACCATACATTGAAGCAGATTGCC	
il1ß fw	GGAGAGGTTAAAGGGTGGCGA	[52]
il1ß rv	TGCCGACTCCAACTCCAACA	
irf3 fw	AACAAGGCATGCAGGGTTCTAAAT	[53]
irf3 rv	ACGTGTGCAATCAGTACCAGC	
irf3 probe	ATGCTCCTTAATATTTTGCTGTGCGACCTGGATT	
irf7 fw	ACAACATCAGCCTGCAGATCTC	TC167655
irf7 rv	CGGGCCAGGTGGCATT	
N_{VHSV} fw	GACTCAACGGGACAGGAATGA	[54]
N_{VHSV} rv	GGGCAATGCCCAAGTTGTT	
N_{VHSV} probe	TGGGTTGTTCACCCAGGCCGC	
tlr9 fw for normal PCR	CCCCTTTGACTGCTCCTGTGACACTT	[20]
tlr9 rv for normal PCR	CATCCCAGCCGTAGAGGTGCCTGAGTAG	
gapdh fw for normal PCR	ATGTCAGACCTCTGTGTTGG	[35]
gapdh rv for normal PCR	TCCTCGATGCCGAAGTTGTCG	

ef1α: elongation factor 1 alpha; *tlr9*: toll-like receptor 9; *mx*: isoform 1 and 3 of the trout antiviral Mx protein; *tnfα*: tumour necrosis factor alpha; *il1ß*: interleukin 1 beta; *irf3*: interferon regulatory factor 3; *irf7*: interferon regulatory factor 7; N_{VHSV}:N protein of VHSV; *gapdh*: glyceraldehyde 3-phosphate dehydrogenase; fw: forward; rv: reverse; PCR: polymerase chain reaction

FIGURE LEGENDS

Figure 1: Percentage of VHSV infectivity as estimated by N_{VHSV} gene expression analysis by real-time PCR. RTG-2 cells were treated with CpG 2133 (CpG1), CpG 2143 (CpG2), CpG 1982 (nCpG) or poly I:C (pIC) (24 hours at 20°C), or transfected with pAE6 or pAE6-G (72 hours at 20°C), and then infected with VHSV with a multiplicity of infection (m.o.i.) of 10^{-2} pfu/cell. Gene expression is normalized against *ef1α* gene and relative to infected untreated cells. Data represent the mean fold changes ± SD for three independent experiments, each performed in duplicate. An asterisk (*) denotes statistically significant differences between the different treatments and the control cells according to a Tukey test.

Figure 2: Constitutive expression of rainbow trout *tlr9* in RTG-2 cells untreated or transfected with pAE6 or pAE6-G, evaluated by normal PCR (A). Transcript levels of *tlr9* and selected immune-related genes in RTG-2 cells treated with CpG ODNs or transfected with pAE6 or pAE6-G, evaluated by real-time PCR (B). *tlr9*, interferon regulatory factors *irf3* and *irf7*, proinflammatory cytokines *il1ß* and *tnfa*, and antiviral *mx* genes transcript levels in RTG-2 cells treated with CpG 2133 (CpG1), CpG 2143 (CpG2), CpG 1982 (nCpG) or poly I:C (pIC) (24 hours at 20°C), or transfected with pAE6 or pAE6-G (72 hours at 20°C). Gene expression is normalized against *ef1α* gene. Data represent the mean fold changes ± SD for three independent experiments, each performed in duplicate. An asterisk (*) denotes statistically significant differences between the different treatments and the control cells according to a Tukey test.

Figure 3: Representative immunofluorescence images of Mx protein expression in RTG-2 cells stimulated with poly I:C or transfected with pAE6-G (72 hours at 20°C). RTG-2 cells micrographs with polyclonal anti-Mx antibody at fluorescent (a) and visible (b) light fields; (c), Merged image of fields a and b.

Figure 4: Effect of the inhibition of endosomal acidification on the transcript levels of *tlr9* and selected immune-related genes in RTG-2 cells treated with CpG ODNs or transfected with pAE6 or pAE6-G. *tlr9*, interferon regulatory factors *irf3* and *irf7*, proinflammatory cytokines *il1ß* and *tnfa*, and antiviral *mx* genes transcript levels in RTG-2 cells pre-treated with 10 mM NH_4Cl for 1hr and then treated with CpG 2133 (CpG1), CpG 2143 (CpG2), CpG 1982 (nCpG) or poly I:C (pIC) (24 hours at 20°C), or transfected with pAE6 or pAE6-G (pAE6-G) (72 hours at 20°C) in the presence of NH_4Cl. Data represent the mean fold changes ± SD for three independent experiments, each performed in duplicate. An asterisk (*) denotes statistically significant differences between the different treatments and the control cells according to a Tukey test.

Figure 5: Time course of recovery of endosomal low-pH after blocking endosomal acidification with NH_4Cl. RTG-2 cells were pre-treated with 10 mM NH_4Cl and then transfected with pAE6-G. NH_4Cl was retired at the following times: before transfection (t 0), a quarter of hour (t 1/4h), half and hour (t 1/2h), 2 (t 2h), 3 (t 3h), 4 (t 4h), 6 (t 6h) and 7 (t 7h) hours after transfection. After that, G_{VHSV} (black square) and *mx* (black circle) genes transcript levels were

measured. Gene expression is normalized against *ef1α* gene and relative to NH_4Cl untreated and non-transfected cells. Data represent the mean fold changes ± SD for three independent experiments, each performed in duplicate. An asterisk (*) denotes statistically significant differences between the different times of NH_4Cl exposure and transfected cells without NH_4Cl, according to a Tukey test.

Figure(s)

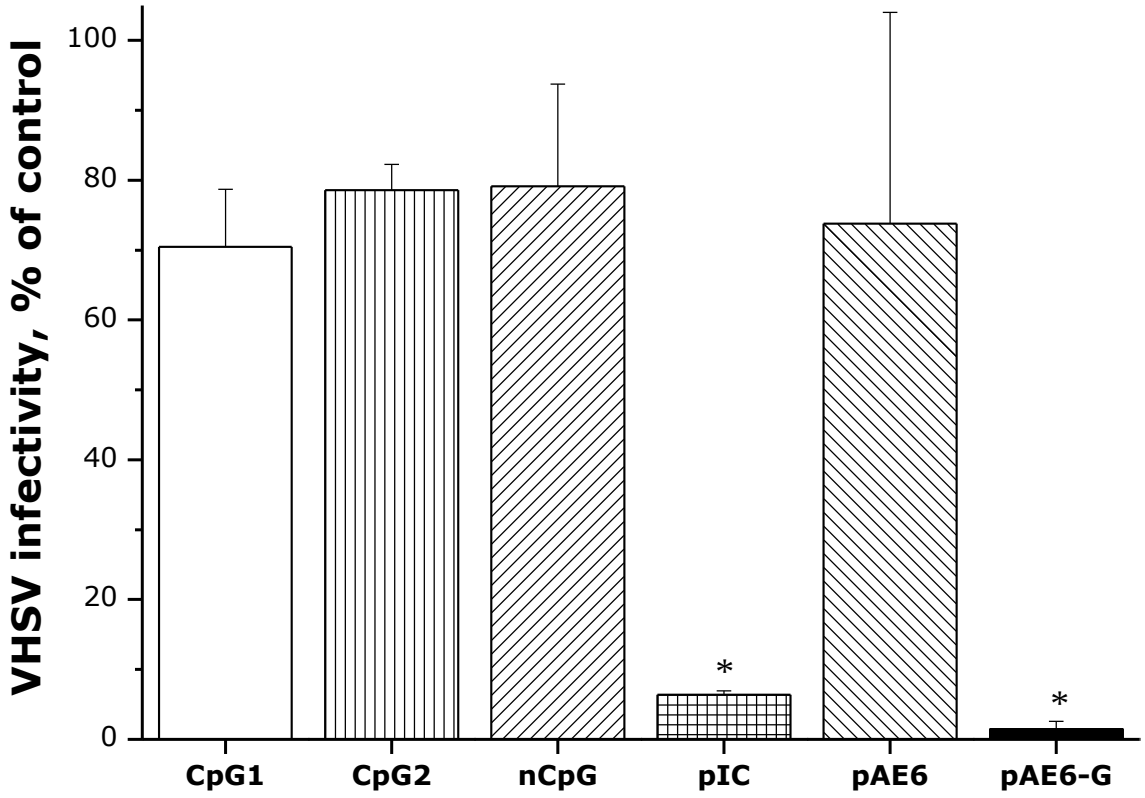
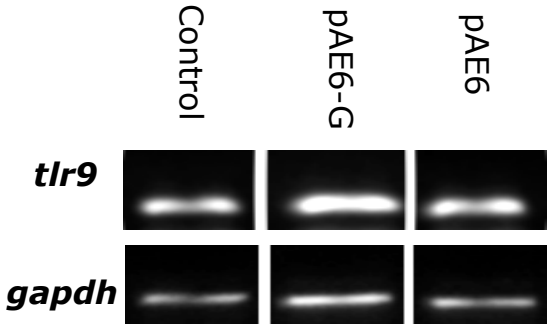
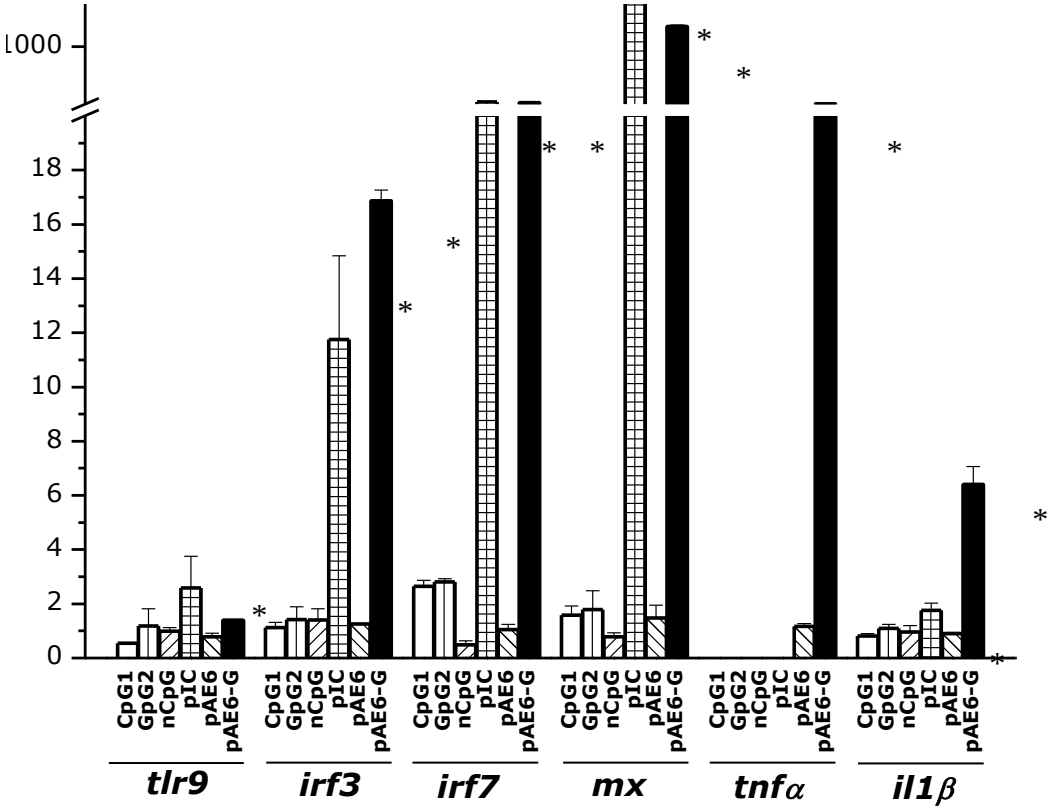


Figure 1.

A


Control
pAE6-G
pAE6
tlr9
gapdh

B


1000
18
16
14
12
10
8
6
4
2
0
CpG1
GpG2
nCpG
pIC
pAE6
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tlr9
irf3
irf7
mx
tnfα
il1β

Figure 2.

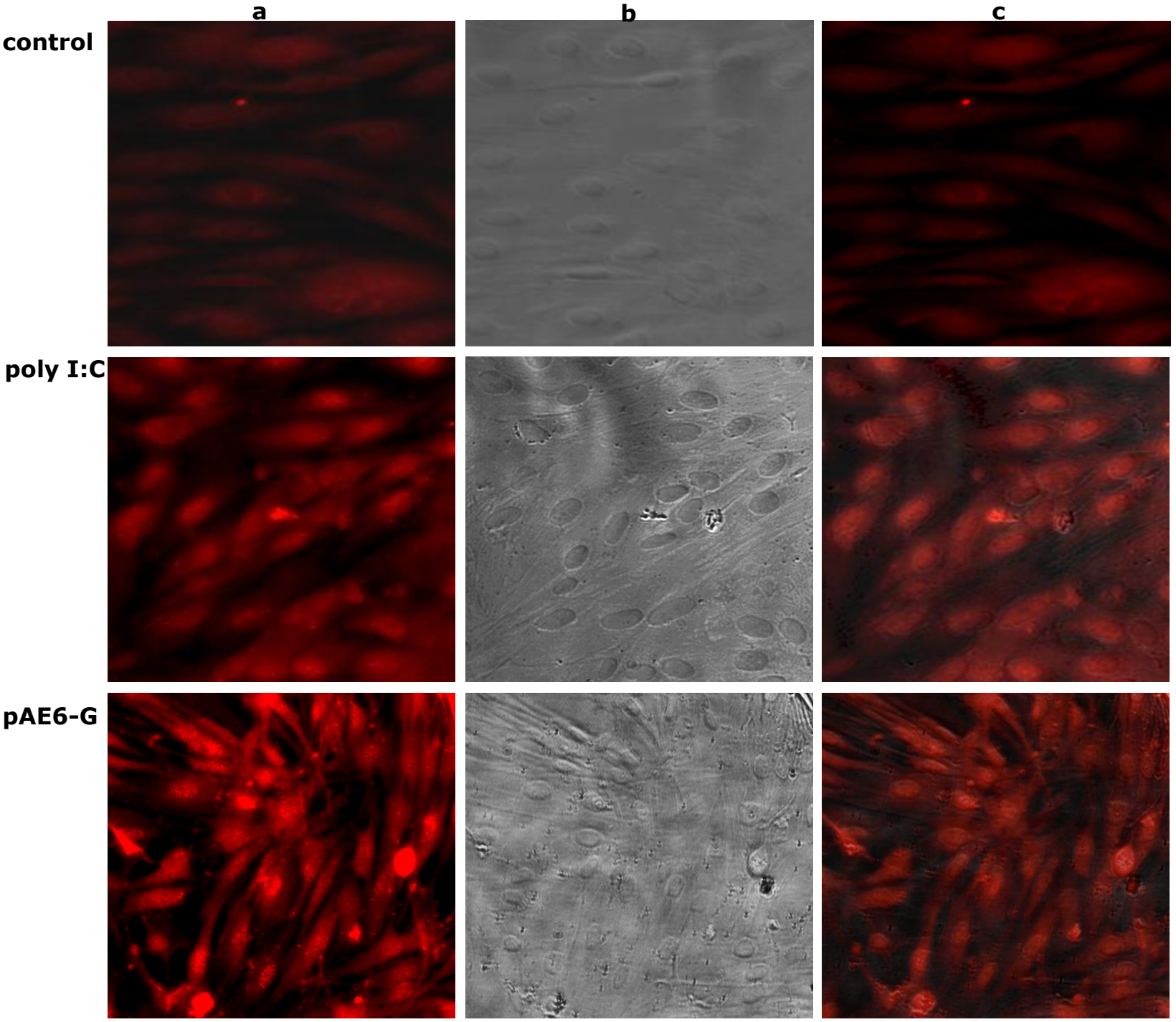
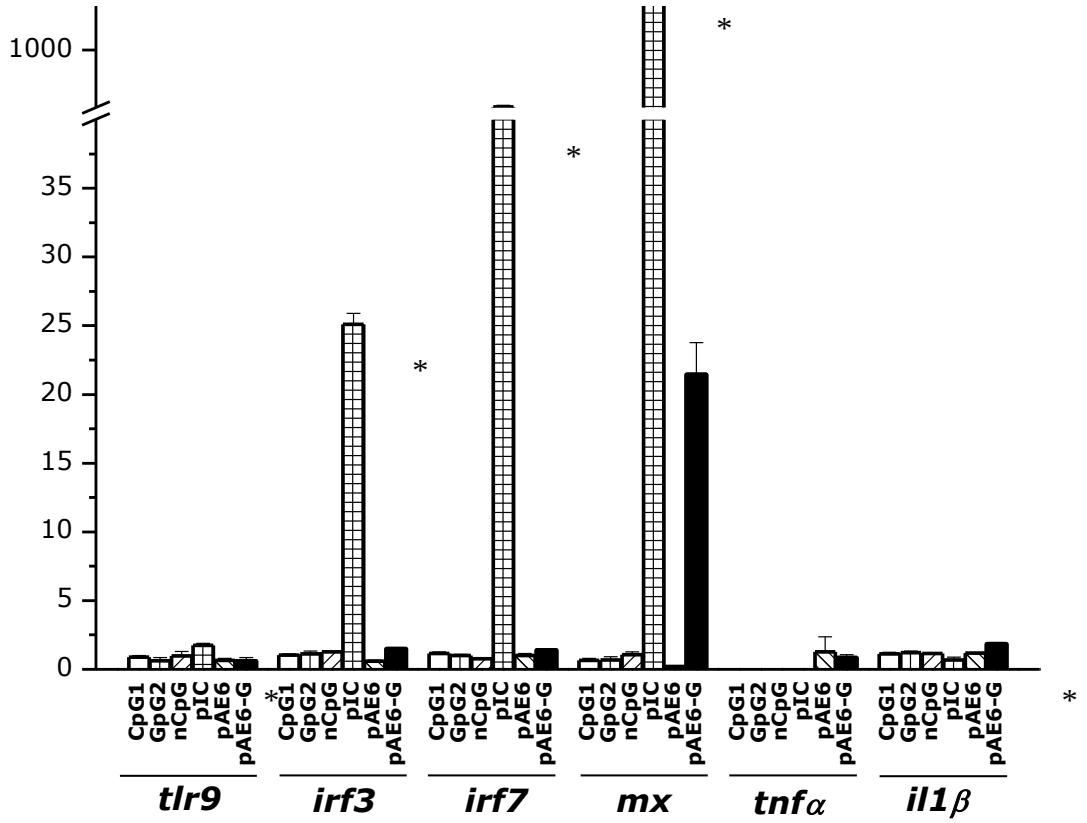


Figure 3.


1000
35
30
25
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CpG1
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nCpG
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tlr9
irf3
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mx
tnfα
il1β
*

Figure 4.

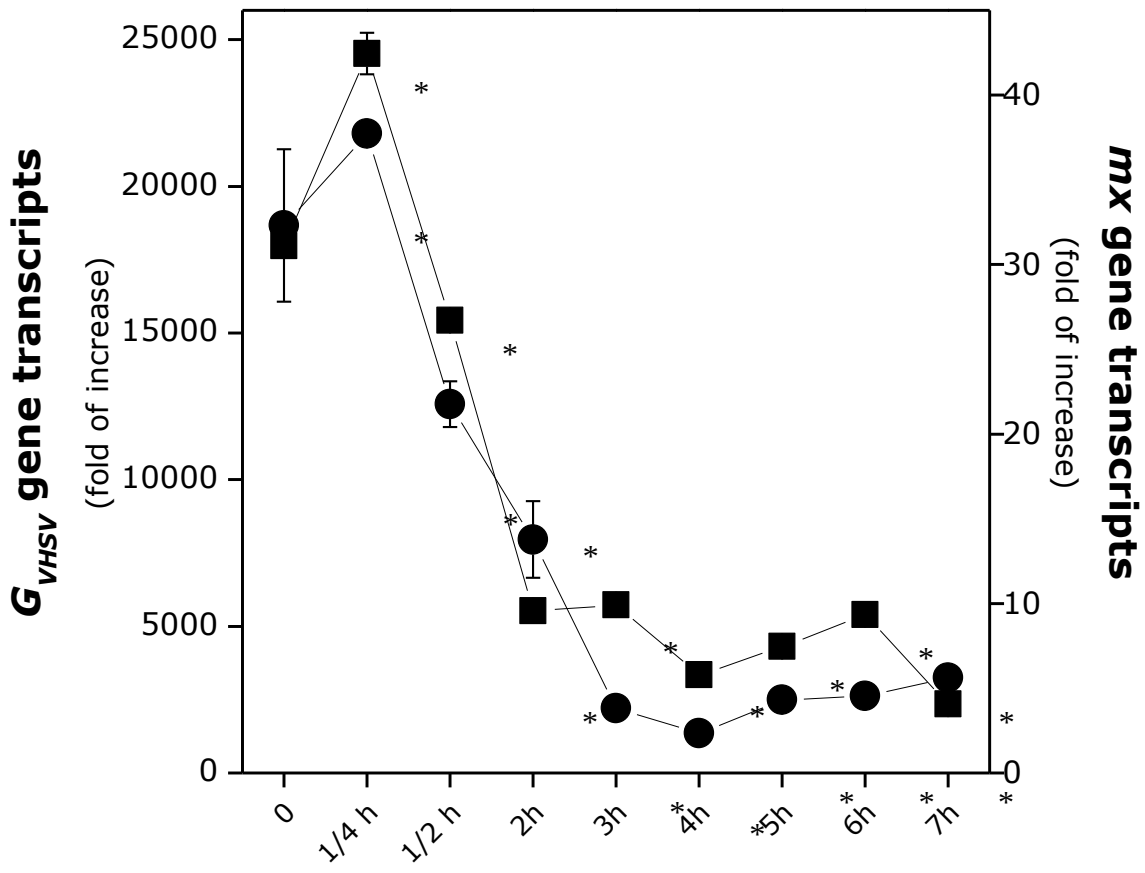
G_{VHSV} gene transcripts
(fold of increase)
mx gene transcripts
(fold of increase)
25000
20000
15000
10000
5000
0
40
30
20
10
0
0
1/4 h
1/2 h
2h
3h
4h
5h
6h
7h

Figure 5.