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Type I interferon gene therapy protects against cytomegalovirus-induced myocarditis

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SUMMARY

Type I interferons (IFNs) are produced early in response to viral infection and modulate adaptive immunity. Previously we demonstrated localized protection against murine cytomegalovirus (MCMV) infection in IFN DNA-inoculated mice. Here we examine the effect of seven IFN subtypes (*IFNA1*, *A2*, *A4*, *A5*, *A6*, *A9* and *B*), administered by DNA inoculation, on systemic MCMV infection and myocarditis. IFN transgene expression altered the pathogenesis of MCMV infection with regard to virus titre and myocarditis. *IFNA6* treatment reduced MCMV replication whilst *IFNA5* and *A2* enhanced virus replication. *IFNA6*, *A9*, and *B* treatment inhibited acute myocarditis. A T helper type 1-like, antibody and cytokine, response correlated with decreased virus titre and myocarditis. In addition, *IFNA6* was able to reduce chronic cardiac inflammation. This research into the effectiveness of seven type I IFNs, using DNA gene therapy, highlights the need for correct subtype usage in the treatment of disease. We demonstrate effective subtypes for treatment in both the acute and chronic phases of MCMV infection and the resultant development of myocarditis.

INTRODUCTION

The type I interferons (IFNs), produced as part of the innate immune response, can facilitate and direct the adaptive immune response. The type I IFNs belong to a multigene family with over 14 IFN-α subtypes in man, over 10 IFN-α subtypes in mouse[1–6] and only one IFN-β subtype in both man and mouse. There is a high degree of homology between the subtypes at the amino acid level with 80–95% homology between the IFN-α subtypes and 50% homology with IFN-β. Furthermore, the murine and human IFN gene families are highly analogous[7,8] with more than 70% homology in nucleotide sequence for the IFN-α subtypes and 68% for the IFN-β subtypes.[9] The IFN subtypes signal via a common receptor, composed of the IFNAR1 and IFNAR2 subunits leading to JAK-STAT activation, the formation of ISGF3 and subsequent onset of gene expression.[10]

Therapeutic properties of type I IFNs include antiviral,[11,12] antiproliferative[13] and immunomodulatory effects.[14] More specifically, IFNs have been noted to regulate major histocompatibility complex (MHC) gene expression and natural killer cell activation and to mediate antibody-dependent cytotoxicity via other cytokines. In addition, the type I IFNs may induce both bystander T-cell proliferation *in vivo* and potentiate the clonal expansion and survival of antigen-specific $CD8^+$ T cells.[15] Furthermore, type I IFNs promote T helper 1 (Th1) type responses, by inhibiting interleukin-4 (IL-4) and IL-5 secretion, increasing IFN-γ production in $CD4^+$ cells,[16,17] and enhancing immunoglobulin M (IgM), IgG2a and IgA, but not IgG1 production in B cells.[18]

Extensive clinical trials have led to licensing of certain type I IFN subtypes for the treatment of several disease conditions including hepatitis, hairy cell leukaemia, condyloma acuminatum, multiple sclerosis and Kaposi's sarcoma.[19–24] Surprisingly, preparations of IFN-α currently available for clinical use are either a single recombinant IFN-α2 subtype (Roferon, Roche, Basel, Switzerland)

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Abbreviations: CMV, cytomegalovirus; H & E, haematoxylin & eosin; IE, immediate-early; IFN, interferon; MCMV, murine cytomegalovirus; p.i., post-infection; SE, standard error; TA, tibialis anterior.

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obtained from transfected *Escherichia coli*, or a mixture of many IFN subtypes obtained from Sendai virus-stimulated human lymphoblastoid cells or primary human blood leucocytes (Glaxo-Wellcome, Durham, NC). However, preparation and purification of IFNs result in loss of subtype diversity, leaving IFN-α2 as the major component.[1] Treatment for multiple sclerosis utilizes recombinant Betaseron (IFN-β-1b) or Avonex (IFN-β-1a).[25,26] Therefore, despite the diversity of the type I IFN subtypes, their use for the treatment of human disease is surprisingly limited.

Cytomegalovirus (CMV) has been implicated in the pathogenesis of myocarditis and dilated cardiomyopathies.[27–32] This insidious disease ranges from a transient inflammation, which is often asymptomatic, to a fulminant syndrome with heart failure, arrhythmia and sudden death. CMV is ubiquitously expressed in the population and generally remains asymptomatic. However, CMV is an important pathogen of the immunocompromised, including transplant recipients, acquired immune-deficiency syndrome (AIDS) patients and neonates. CMV infection has been implicated in 40–50% of cardiomyopathies and in 17–30% of patients with myocarditis.[33] We have established an experimental mouse model using murine cytomegalovirus (MCMV) in order to gain insight into the interaction between virus and the immune response in the development of myocarditis.[34,35] BALB/c mice are susceptible to the development of MCMV-myocarditis with resultant cardiovascular disease characterized by an acute and chronic phase. Accumulating evidence indicates that the chronic phase of myocarditis is autoimmune, with the production of specific autoantibodies to the S2 region of the heavy chain of cardiac myosin.[36,37] We have also observed that neutralizing monoclonal antibodies raised against structural MCMV proteins exhibit cross-reactivity with cardiac myosin.[38] Such cross-reactive autoantibodies have been demonstrated to play an immunopathogenic role in myocarditis.[38] The development of myocarditis is T-cell-dependent,[39] involving both $CD4^+$ and $CD8^+$ T cells.[40] Surprisingly, very low titres of infectious virus are found in the heart, with foci of inflammatory cells not necessarily co-localized to virus-infected cells. Nevertheless, virus remains latent in heart tissue, with detection of early (*ie1*) and late (*gB*) genes and transcripts for *ie1* present to day 100 post-infection (p.i.).[41]

Previously we found murine type I IFN-α1, -α4 and -α9 DNA expression in the tibialis anterior (TA) muscle of mice reduced virus replication upon inoculation of MCMV at this site.[42,43] Strikingly, intramuscular IFN transgene expression reduced the number of foci of inflammatory cell infiltrates in virus-inoculated muscle, establishing the effectiveness of IFN expression when localized with virus. Here, we analyse the efficacy of IFN transgene expression, on systemic murine MCMV infection. We examine the effectiveness of IFN subtypes -α1, -α2, -α4, -α5, -α6, -α9 and -β on virus replication, cardiac inflammation, antibody isotype response and cytokine profile. Data indicate that a constitutive low level of IFN transgene expression was sufficient to modify both tissue virus load as well as acute- and chronic-phase myocarditis. Notably, *IFNA6* gene therapy reduced virus load in all target tisues examined. Acute-phase myocarditis was reduced with *IFNA6*, *A9* and *B* transgene expression, whilst *IFNA6* alone reduced chronic-phase myocarditis. Our results have profound implications with regard to choice of IFN subtype for treatment of viral infection and the use of naked DNA therapy for constitutive expression of cytokines.

MATERIALS AND METHODS

Mice

Specific pathogen-free male BALB/c mice (4 weeks old) were purchased from the Animal Resources Centre (Murdoch, Western Australia).

Virus

The K181 strain of MCMV (originally obtained from D. Lang, Duke University, Durham, NC) was prepared as a salivary gland homogenate from virus-infected weanling BALB/c mice, and stored in liquid nitrogen, as described elsewhere.[38] Virus titres in infected mice were quantified by plaque assay and calculated as mean plaque-forming units (PFU)/g of tissue.

Expression plasmid constructs

The mammalian expression vector, pkCMVint, was kindly provided by VICAL (San Diego, CA). This vector contains the human CMV immediate-early (IE) 1 gene enhancer/promoter and human CMV intron A for transcription initiation coupled with the simian virus-40 polyadenylation signal. All gene inserts include the sequence for the signal peptide located 69 nucleotides upstream of the first cysteine TGT codon of the mature protein. The IFN genes were amplified by polymerase chain reaction (PCR) using liver tissue from BALB/c mice and contained 10–25 nucleotides upstream of the first ATG start codon and 10–24 nucleotides downstream of the TGA stop codon. The full-length murine *IFNA1*, *A2*, *A4*, *A5*, *A6*, *A9*, and *B* genes were subcloned into the pkCMVint expression vector via gene amplification using specific primers in the PCR. Fragments incorporated were IFNA1, −21 to +525 bp; IFNA2, −21 to +596 bp; IFNA4, −21 to +584 bp; IFNA5, −18 to +593 bp; IFNA6, −24 to +590 bp; IFNA9, −25 to +595 bp; and IFNB, −10 to +599 bp. All inserts were sequenced in both directions to ensure complete integrity. Large-scale plasmid preparations were obtained from terrific broth cultures of transformed *E. coli* (DH-5α) using standard DNA extraction procedures with LiCl precipitation. Plasmid integrity was checked by agarose gel electrophoresis and concentrations determined by spectrophotometric analysis.

IFN plasmid transfections

Transfection with the IFN plasmid constructs was performed with calcium phosphate precipitation as previously described.[44] Briefly, COS-7 cells were grown to 70% confluency and 20 μg DNA resuspended in 0·5 ml $CaCl_2$ and an equal volume of 2 × BBS [50 mM *N*,*N*-bis (hydroxyethyl)-2-aminoethanesulphonic acid (BES),

280 mM NaCl, 1·5 mM Na_2HPO_4, pH 6·95] added dropwise with aeration to the cultures. After 24 hr, the cells were washed twice and overlaid with a minimal amount of Dulbecco's modified Eagle's minimal essential medium/10% fetal calf serum. Supernatants were collected at 24 hr, filtered (Millipore 0·22 μm) and acid treated (pH 2·0) to remove acid-labile proteins.

Vaccination protocols

To induce muscle regeneration, mice were injected bilaterally in the TA muscles with 20 μl of 0·5% bupivacaine 5 days before inoculation with DNA constructs. Mice were inoculated with 100 μg of DNA plasmids in a 25-μl volume of pyrogen-free saline bilaterally in the TA muscles. Two weeks post-DNA inoculation, mice were injected with 100 μl volume of MCMV (10^4 PFU/mouse) diluted in pyrogen-free saline by the intraperitoneal (i.p.) route (five mice/group).

IFN bioassay

Standard murine type I IFNs (Lee Biomolecular Inc., San Diego, CA) and test samples were titrated in an IFN bioassay as previously described.[42] Briefly, individual tissue homogenates and sera from mice were evaluated for acid-stable IFN titres using 50% protection from encephalomyocarditis virus-induced cytopathic effect (CPE) of L929 cell monolayers. Titres are expressed as mean IU/g of tissue or IU/ml sera ±standard error (SE).

Virus quantification

Virus titres were quantified for liver and spleen taken at day 3 p.i. and for salivary gland tissues at days 7, 30 and 56 p.i., as determined by plaque assay using mouse M2-10B4 stromal cells. Virus titres are expressed as mean PFU/g tissue ±SE (five mice/group). The limit of detection was 100 PFU/g liver and spleen and 6400 PFU/g salivary gland tissue.

Myocarditis histology

Hearts from experimental mice were taken at days 7, 30 and 56 p.i., transected along the midline, fixed in Bouin's fluid and processed as paraffin-embedded blocks. Heart sections stained with haematoxylin & eosin (H & E) were examined microscopically for evidence of cellular inflammation and necrosis. Two heart sections were scored for each animal. Myocarditis was evaluated histologically as the mean number of inflammatory foci per heart section ±SE (five mice/group).

MCMV and cardiac myosin enzyme-linked immunosorbent assays (ELISAs)

MCMV and cardiac myosin (BALB/c origin) were used as antigens in the ELISA as previously described.[38] Antibody responses were evaluated by ELISA and titres were expressed as $\log_2$ serum antibody titre ±SE. The limit of detection was 2·0 $\log_2$. The cytokines, IFN-γ, IL-2, IL-4, IL-6, IL-10 and IL-18 were quantified in the sera using ELISA kits (OptEIA ELISA, Pharmingen, San Jose, CA). Murine IFN proteins were quantified using an IFN-α ELISA kit (PBL, New Brunswick, NJ).

Statistical analysis

Levels of significance ($P < 0{\cdot}05$) were determined by the unpaired Student's *t*-test assuming unequal variance between the means. All experiments were performed at least twice with five mice/group, unless otherwise stated.

RESULTS

Expression of type I IFN DNA subtypes *in vitro* and *in vivo*

The seven type I IFN DNA constructs were designed for consistency in stability and expression levels of mRNA transcripts. Primers were designed 10–25 nucleotides up- and down-stream of the start and stop codons to allow for expression driven by the pkCMVint promoter and incorporation of the vector polyA signal (Fig. 1).

To ensure production of biologically active protein, blank vector (vehicle) and type I IFN plasmid constructs were transfected into COS-7 cells in a serum-free environment and the supernatants were harvested and acid-treated for collection of IFN acid-stable product. Supernatants were subjected to IFN bioassay for reduction in encephalomyocarditis virus (EMCV)-induced CPE of L929 cells. Subtype expression was detected for IFN-α1 (625 IU/ml), IFN-α2 (156 IU/ml), IFN-α4 (1250 IU/ml), IFN-α5 (5000 IU/ml), IFN-α6 (2500 IU/ml), IFN-α9 (5000 IU/ml) and IFN-β (2500 IU/ml), as compared to the international murine IFNα/β standard (Lee Biomolecular Inc., CA). Confirmation of type I IFN protein was determined by IFN-α ELISA using supernatants from transfected COS cells and the international murine IFN-α/β standard. IFN protein was detected in alpha subtypes IFN-α1 (2583 pg/ml), IFN-α2 (1549 pg/ml), IFN-α4 (1294 pg/ml), IFN-α5 (3111 pg/ml), IFN-α6 (371 pg/ml), and IFN-α9 (94 pg/ml), with no detection of IFN-β as expected. Standard international murine IFN-α/β protein mixture at 1000 IU/ml (approximately 85% IFN-β) equated to 255 pg/ml as determined by ELISA. The finding that murine IFN subtypes differ significantly in their specific activity has been noted previously,[45] and similarly differential activities have been observed in humans.[1] Thereby, the *in vitro* activity of IFN protein expression was established.

The activity of individual IFN transgene expression was next determined *in vivo*. IFN constructs were injected bilaterally into the TA muscle of mice, 2 weeks later mice were infected with MCMV i.p. and sera (day 3) and TA muscles (day 7) were harvested. Biologically active IFN was detected by IFN bioassay in both the sera and TA muscles. Typically, IFN activity in the sera ranged fourfold from approximately 10 to 40 IU/ml (Table 1). In the TA muscles, IFN was detected in all mice and ranged threefold from 13 000 to 40 000 IU/g muscle. In virus-infected mice receiving the vehicle without the IFN transgene, biologically active IFN could not be detected in either sera or TA muscle. In addition, *IFNA6* transgene expression in uninfected mice produced biologically active IFN in sera (30·0 IU/ml) and the TA muscle (15 500 IU/ml) confirming the endogenous activity of the plasmid DNA constructs. Importantly, this

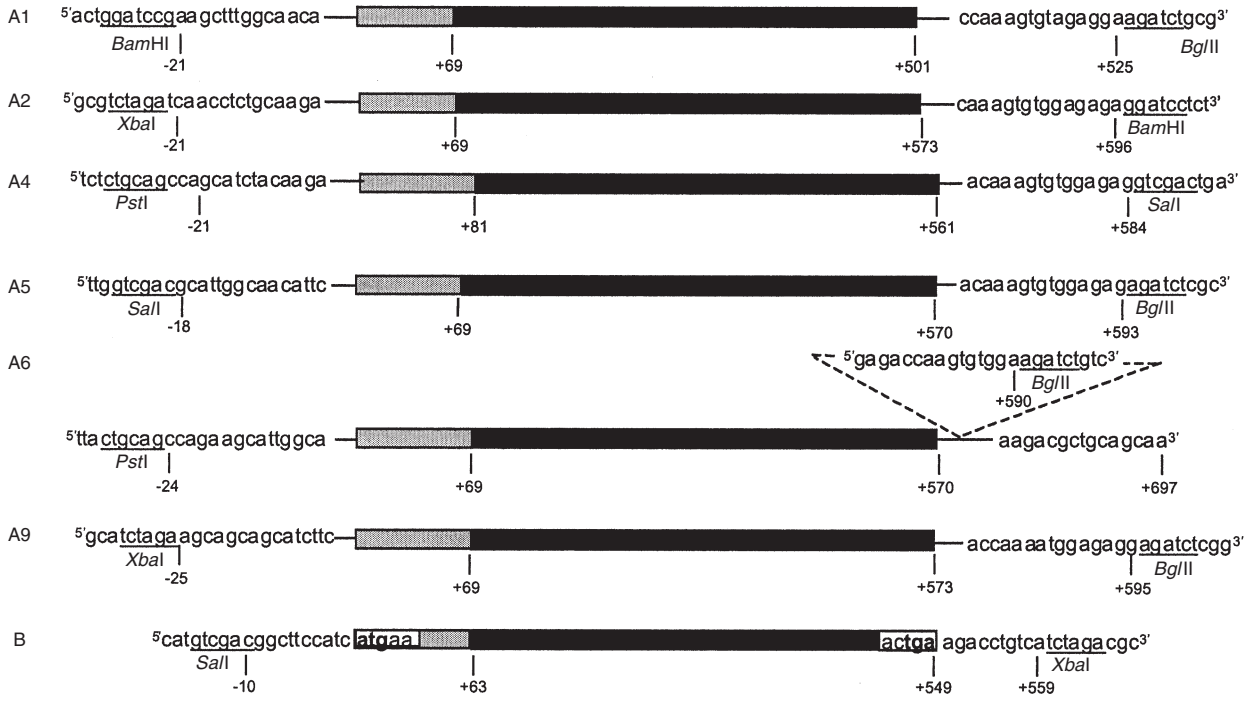

Figure 1. IFN transgenes expressed in the mammalian expression vector pkCMVint. *MuIFNA1*, *A2*, *A4*, *A5*, *A6*, *A9* and *B* carrying the full-length MuIFN subtype genes (black) including the signal sequence (shaded grey) located upstream of the mature protein. Flanking primer sequences with incorporated restriction enzyme sites used for subcloning of each transgene cassette are shown.

Table 1. Interferon activity in sera and TA muscle of mice following DNA injection and viral challenge

IFN subtype*	Sera (IFN IU/ml)	TA muscle ($\times 10^3$ IU/g)
Vehicle	0·0 ± 0·0 (5/5)	0·0 ± 0·0 (5/5)
pkCMVint.IFNα1	18·2 ± 6·9 (3/5)	35·3 ± 14·8 (5/5)
pkCMVint.IFNα2	15·6 ± 4·4 (5/5)	40·0 ± 14·3 (5/5)
pkCMVint.IFNα4	25·0 ± 2·9 (5/5)	15·9 ± 4·6 (5/5)
pkCMVint.IFNα5	41·0 ± 13·3 (4/5)	13·0 ± 4·0 (5/5)
pkCMVint.IFNα6	10·4 ± 2·6 (3/5)	16·9 ± 3·6 (5/5)
pkCMVint.IFNα9	18·2 ± 2·6 (3/5)	16·2 ± 3·6 (5/5)
pkCMVint.IFNβ	30·2 ± 0·0 (2/5)	15·6 ± 3·7 (5/5)

*DNA (200 μg) was injected bilaterally into regenerating TA muscles of adult mice 5 days after bupivacaine treatment. Fourteen days following DNA injections mice were challenged with 1×10^4 PFU MCMV i.p., sera were harvested at day 3 and TA muscle was harvested at day 7. Data are expressed as mean ± SEM; the number of mice testing positive is give in parentheses.

established the expression of IFN product at both localized and systemic sites.

Type I IFN DNA treatment reduces systemic MCMV replication

The effects of type I IFN subtypes on reduction of virus replication in target organs distal to the DNA-inoculated muscle, namely the spleen, liver and the salivary glands, were examined next. Mice were immunized intramuscularly with plasmid expressing individual type I Mu*IFN* subtypes or vehicle 2 weeks prior to challenge with infectious MCMV. Early virus replication was monitored in the spleen and liver (day 3 p.i.), and in the salivary gland (day 7 p.i.) (Fig. 2). In the spleen, virus titre was markedly reduced with *IFNA2*, and significantly reduced with *IFNA6* treatment. Conversely, *IFNA5* treatment significantly increased CMV replication in the spleen. In the liver, virus replication was significantly reduced with *IFNA6* treatment. Virus replication in the salivary glands was found to be significantly reduced in mice treated with the subtypes *IFNA5* and IFN*A6*. Notably, treatment with the IFN subtypes had differential protective effects against virus replication in each tissue type examined.

Type I IFN DNA treatment immunomodulates myocarditis

In order to evaluate the therapeutic effects of naked DNA delivery of Mu*IFN* genes on myocarditis, hearts were taken at day 7 in the experimental mice described above. Hearts stained with H & E were examined histologically for myocarditis (Fig. 3). Hearts from normal, uninfected mice did not show any evidence of myocarditis. Mice receiving the vehicle and infected with MCMV for 7 days showed acute myocarditis with foci characterized by a predominantly mononuclear cell infiltrate and necrosis of adjacent

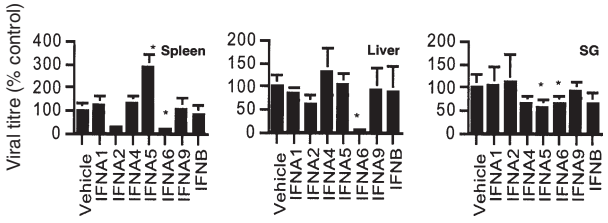

Figure 2. Effect of *IFN* DNA treatment on MCMV replication *in vivo*. MCMV titres in the spleen (day 3 p.i.), liver (day 3 p.i.), and salivary glands (SG) (day 7 p.i.). Mice were inoculated with 10^4 PFU MCMV i.p. at 2 weeks post-DNA vaccination. Virus titres are expressed as percentage control titre (average PFU/g tissue, five mice per time-point $\pm$SE) found for mice vaccinated with the vehicle. *Statistically significant differences between treatment groups and vehicle ($P<0{\cdot}05$) are shown and are representative of two independent experiments.

myofibres. Treatment of mice with *IFNA6*, *A9*, and *B* significantly reduced the number of foci in the acute phase of myocarditis. Additionally the number of infiltrating cells per focus was reduced by treatment with *IFNA6* and *B*. Treatment with *IFNA2* and *A4* marginally increased the number of foci per heart section. Treatment with *IFNA1* and *IFNA5* did not significantly alter the severity of acute myocarditis.

Type I IFN DNA treatment influences antibody isotype response in MCMV infection

During the acute phase of myocarditis, an antibody response is directed both at viral antigens and the self-antigen, cardiac-myosin, in MCMV-infected mice. We found that virus load did not always correlate with the subsequent development of myocarditis, therefore we

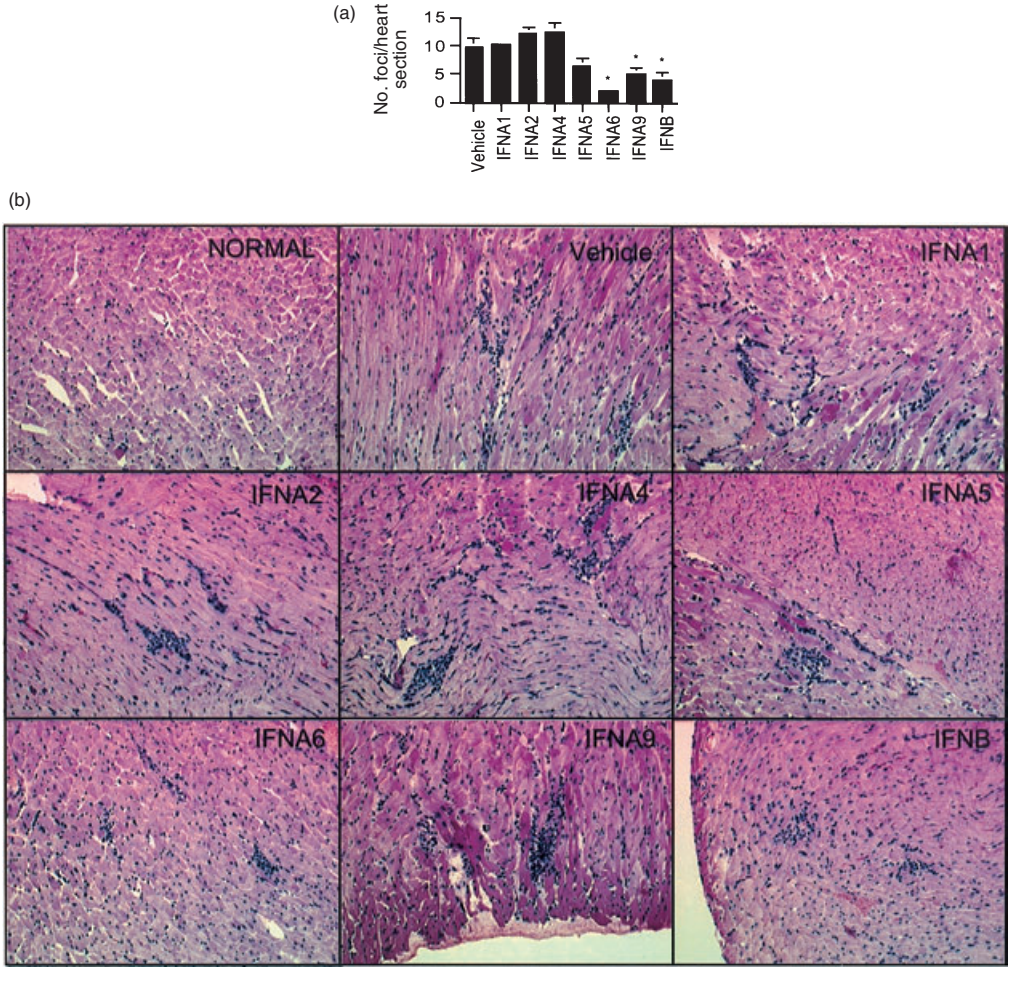

Figure 3. DNA treatment with *IFNA6*, *IFNA9* and *IFNB* reduces MCMV-induced myocarditis. BALB/c mice were inoculated with 10^4 PFU of MCMV i.p. at 2 weeks post-DNA vaccination. (a) The average number of inflammatory foci/heart section from groups of five mice per time-point $\pm$SE are shown at day 7. *Statistically significant differences between treatment groups and vehicle ($P<0{\cdot}05$) are shown and are representative of two independent experiments. (b) Histopathology of murine cardiac tissue (H & E stained, $\times$160). Heart sections for normal or vehicle, *IFNA1*-, *IFNA2*-, *IFNA4*-, *IFNA5*-, *IFNA6*-, *IFNA9*- and *IFNB*-treated, MCMV-infected mice (day 7 p.i.) are as indicated.

examined anti-MCMV and anti-cardiac myosin antibodies. The titres of IgG1 and IgG2a antibodies reactive with either MCMV or cardiac myosin were determined by ELISA using individual sera from mice infected with virus for 7 days. The antiviral antibody titres were predominantly IgG1 for vehicle and the majority of IFN subtype-treatment groups (Table 2). Notably, a significantly lower titre was found for IgG1 antibodies in the groups treated with *IFNA6* and *B* subtypes. To our surprise, *IFNA9* treatment elicited high levels of antiviral IgG2a antibodies in the sera of treated mice. It is speculative to suggest that such antibodies may compete with the antiviral activity of the induced IgG1 antibodies.

The level of anticardiac myosin autoantibodies were examined next. Predominantly, IgG1 antibody isotype responses were detected in all groups and, similar to anti-MCMV antibodies, the lowest titres were observed for *IFNA6*- and *B*-treated mice. Therefore, with regard to *IFNA6* and *B* treatment, a lower titre of IgG1 antibodies against MCMV and cardiac myosin corresponded with a reduction in the number of foci of inflammatory infiltrates observed in the heart during acute-phase myocarditis.

Type I IFN alters cytokine profiles following MCMV challenge

Cytokine production in response to viral infection skews the immune response and ultimately determines the effectiveness of the adaptive immune response. We investigated the profile of Th1- (IFN-γ, IL-2 and IL-18) and Th2- (IL-4, IL-6 and IL-10) like cytokines in mice pretreated with IFN and challenged with MCMV. Cytokine production in sera was measured on day 7 p.i. by ELISA using individual samples from mice (Fig. 4). During the acute phase of infection, *IFNA6* treatment significantly stimulated IFN-γ production, with an increase in IFN-γ also observed for *IFNA1*, *A5*, *A9* and *B* treatment. Levels of IFN-γ were significantly reduced with *IFNA2* and partial reduction was observed with *IFNA4* treatment. Concurrently, IL-4 titres for *IFNA9*- and *B*-treated groups were decreased, whilst all other subtype-treated groups displayed elevated IL-4 levels. Cumulatively, this suggests a predominant Th1-like response in animals protected from viral myocarditis.

IL-6 production was significantly increased with *IFNA4* treatment and elevated levels were also observed for *IFNA1*, *A5* and *A6* treatment. This is consistent with elevated IL-4 production in groups treated with these subtypes. IL-10 levels were increased in mice treated with *IFNA2*, perhaps explaining the suppression of IFN-γ production. Within the limits of detection by ELISA, no change in IL-2 or IL-18 was observed in any treatment group. Therefore, during the acute phase of myocarditis, type I IFN subtypes exhibited differential production of circulating cytokines, with protective subtypes skewed more towards a Th1-like response.

Table 2. IgG1/IgG2a antibody production against MCMV and myosin is affected by type I IFN treatment

	Anti-MCMV antibody		Anti-myosin antibody	
IFN subtype*	IgG1	IgG2a	IgG1	IgG2a
Vehicle	6·8 ± 0·1	2·0 ± 0·0	4·0 ± 0·0	2·2 ± 0·1
pkCMVint.IFNα1	6·8 ± 0·2	2·0 ± 0·0	3·8 ± 0·2	2·4 ± 0·2
pkCMVint.IFNα2	6·2 ± 0·2	2·0 ± 0·0	3·4 ± 0·2	2·2 ± 0·2
pkCMVint.IFNα4	7·0 ± 0·0	2·0 ± 0·0	3·8 ± 0·2	2·2 ± 0·2
pkCMVint.IFNα5	6·0 ± 0·0†	2·0 ± 0·0	3·8 ± 0·2	2·4 ± 0·2
pkCMVint.IFNα6	5·6 ± 0·2‡	2·0 ± 0·0	3·0 ± 0·4	2·0 ± 0·0
pkCMVint.IFNα9	7·0 ± 0·0	7·0 ± 0·0‡	4·6 ± 0·2	2·0 ± 0·0
pkCMVint.IFNβ	5·8 ± 0·2‡	2·0 ± 0·0	2·8 ± 0·2‡	2·0 ± 0·0

*DNA (200 μg) was injected bilaterally into regenerating TA muscles of adult mice 5 days after bupivacaine treatment. Fourteen days following DNA injections mice were challenged with 1×10^4 PFU MCMV i.p. and sera were harvested at day 7. Data are presented as $\log_2$ serum antibody titre ± SEM for IgG1 and IgG2a.

†$P \leq 0{\cdot}05$.

‡$P \leq 0{\cdot}01$.

Differential effects of IFN DNA therapy in chronic-phase MCMV infection

The establishment of beneficial and harmful effects of IFN subtypes in the acute phase of disease, harboured hope for their use in protection against the chronic phase of disease. This phase of disease is characterized by chronic inflammation in the heart and absence of infectious virus in target tissues.

In the chronic phase of myocarditis we examined an early (day 30) and late (day 56) stage in the development of cardiac inflammation (Fig. 5). *IFNA6* was most effective in reducing cardiac inflammation in the early and late stages of chronic disease, with partial protection afforded by *IFNB*. The salivary gland, a tissue characterized as a site for MCMV persistence, was also examined for virus replication. Surprisingly, treatment with either *IFNA2* or *B* significantly increased the level of persistent infectious virus in the salivary glands at day 30 p.i., virus in the salivary gland was

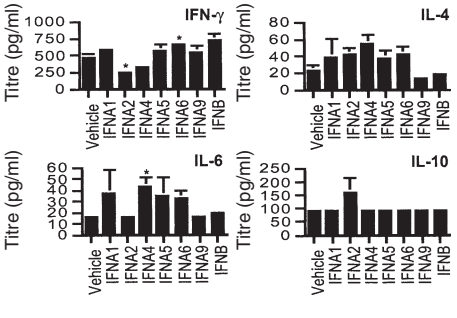

Figure 4. Cytokine expression in sera of *IFN* DNA-treated and MCMV-infected mice. Mice were inoculated with 10^4 PFU MCMV i.p. at 2 weeks post-DNA vaccination. The cytokines IFN-γ, IL-4, IL-6 and IL-10 were determined in the sera at day 7 p.i. by ELISA and are expressed as the average serum titre (pg/ml) ± SE (five mice per group). *Statistically significant differences between treatment groups and vehicle ($P < 0{\cdot}05$) are shown.

cleared in all treatment groups by day 56 p.i. These data highlight a double-sided nature of IFN treatment where IFN subtypes that are effective at reducing disease may be allowing virus particles to escape and thus contributing to virus replication.

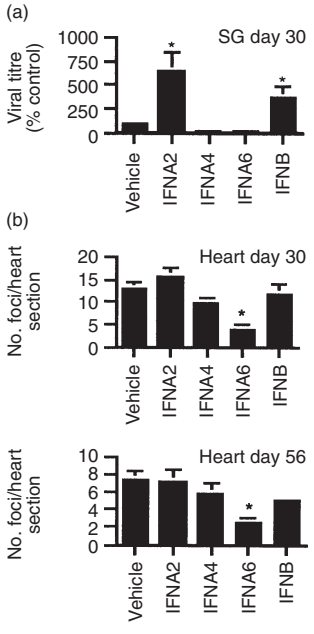

Figure 5. Effect of *IFN* DNA treatment in chronic phase of MCMV infection. BALB/c mice were inoculated with 10^4 PFU of MCMV i.p. at 2 weeks post-DNA vaccination. (a) MCMV titre in the salivary glands (SG) of mice was determined day 30 p.i. Virus titres are expressed as percentage control titre (average PFU/g tissue, five mice per time point ±SE) found for mice vaccinated with the vehicle. (b) The average number of inflammatory foci/heart section from groups of five mice per time-point ±SE are shown at day 30 and day 56. *Statistically significant differences between treatment groups and vehicle ($P<0{\cdot}05$) are shown and are representative of two independent experiments.

During the chronic phase of disease, mice treated with IFN DNA subtypes were examined for IgG1 and IgG2a antibody isotype production. In the onset of chronic disease (day 30), anti-MCMV antibody titre for either IgG1 or IgG2a did not vary amongst the treatment groups (Table 3). Interestingly, at the peak of chronic disease (day 56) anti-MCMV IgG1 antibodies were elevated with both *IFNA6* and *B* treatment. Anti-cardiac myosin IgG1 antibodies, in the early phase of chronic myocarditis, were significantly reduced with *IFNA6* treatment. At the peak of chronic myocarditis, all IFN subtypes tested (*A2*, *A4*, *A6*, and *B*) significantly suppressed the production of IgG1 and IgG2a anticardiac myosin antibodies. Therefore, the IFN subtype affording the best protection against chronic myocarditis (*IFNA6*) induced a lower titre of anticardiac myosin antibodies during the onset of chronic myocarditis.

DISCUSSION

In this report, we show that IFN gene therapy with defined subtypes is effective in the control of MCMV replication and associated cardiac disease. BALB/c mice inoculated i.p. with a sublethal dose of infectious MCMV develop acute viral infection as well as acute- and chronic-phase myocarditis.[38] Previously, we reported that IFN DNA subtypes (*A1*, *A4* and *A9*) reduced localized MCMV replication in the TA muscle.[42,43] The present study examines the effect of low-level, persistent IFN expression using a panel of seven IFN subtypes (*A1*, *A2*, *A4*, *A5*, *A6*, *A9* and *B*) on systemic MCMV infection. We report that delivered IFN genes were capable of exerting antiviral effects on sites of virus replication distal to the DNA inoculation site. In particular, *IFNA6* treatment reduced virus load in all target tissues examined. Somewhat surprisingly, acute myocarditis was reduced with *IFNA6*, *A9* and *B* treatment, despite no alterations in virus load in target tissues for *IFNA9*- and *B*-treated mice. These results support our theory that acute myocarditis is not reflective of virus load but of immunomodulatory responses to viral infection.[40]

Table 3. IgG1/IgG2a antibodies to MCMV and myosin at day 30 and day 56

	Anti-MCMV antibody				Anti-myosin antibody			
	Day 30		Day 56		Day 30		Day 56	
IFN subtype*	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a
Vehicle	6·8±0·2	6·0±0·0	6·2±0·2	10·2±0·4	5·6±0·2	6·2±0·5	4·4±0·2	7·4±0·5
pkCMVint.IFNα2	7·0±0·0	6·0±0·0	6·2±0·2	10·2±0·6	3·3±0·7	6·0±0·6	3·3±0·4†	5·2±0·4†
pkCMVint.IFNα4	7·0±0·0	6·0±0·0	6·6±0·2	10·8±0·2	4·4±0·7	7·0±0·3	3·0±0·3†	5·0±0·4†
pkCMVint.IFNα6	6·8±0·2	6·2±0·2	7·2±0·2‡	9·8±0·4	3·4±0·2†	5·4±0·4	3·4±0·2†	4·8±0·2†
pkCMVint.IFNβ	6·8±0·2	6·2±0·4	7·0±0·0†	10·4±0·2	5·0±0·5	5·8±0·6	2·8±0·2†	4·8±0·4†

*DNA (200 μg) was injected bilaterally into regenerating TA muscles of adult mice 5 days after bupivacaine treatment. Fourteen days following DNA injections mice were challenged with 1×10^4 PFU MCMV i.p., sera were harvested at day 30 and day 56. Data are presented as $\log_2$ serum antibody titre±SEM for IgG1 and IgG2a.

†$P\geq0{\cdot}05$.

‡$P\geq0{\cdot}01$.

IFNA6 and *B* treatment elicited a lower titre of IgG1 antibodies against both MCMV and cardiac myosin which corresponded to a reduction in acute-phase myocarditis. Furthermore, anticardiac myosin IgG1 antibodies were reduced in the early stage of chronic myocarditis with *IFNA6* treatment. It is postulated that expression of IFN-α6 may delay the onset of chronic myocarditis through inhibition of virus replication, via an IFN-γ-mediated pathway, with resultant suppression of anticardiac myosin antibodies. We have also demonstrated that *IFNA9* treatment increased antiviral IgG2a antibodies during the acute phase of disease. *IFNA9* treatment may afford protection via induction of a novel antiviral IgG2a antibody response which competes with the normal production of antiviral IgG1 antibodies in response to MCMV infection.

Possibly, a Th1-like response to viral infection reduces the severity of myocarditis. Elevated circulating levels of IFN-γ production were detected during the acute phase of viral infection in mice with *IFNA6* treatment, whilst IL-4 production was reduced in *IFNA9*- and *B*-treated animals. Indeed, Type I IFNs promote a Th1-like response via increased IFN-γ and inhibition of IL-4 production in $CD4^+$ T cells.[16,17] Furthermore, studies have shown that severe myocarditis develops following viral infection of $IFN\gamma R^{-/-}$ BALB/c mice[46] with shedding of infectious virus up to 6 months p.i. In other models, low levels of IFN-γ prove moderately effective in reduction of viral myocarditis, whereas, high doses of IFN-γ increased morbidity and mortality.[47] In addition, CMV infection can inhibit IFN-γ-induced, MHC class II-dependent antigen presentation,[48] whilst IFN-γ treatment inhibits the reactivation of virus from latency.[49] Potentially, IFN-α6 may stimulate IFN-γ activation of antigen presentation and thus suppress viral reactivation.

IL-6, a pro-inflammatory cytokine, was elevated with *IFNA4* treatment and increases in IL-6 were also observed for *IFNA1*, *A5* and *A6* treatment. Research in IL-6 expression has been indicated to affect adversely the development of myocarditis in other viral systems.[50,51] However, administration of IL-6 prior to viral infection has been shown to reduce EMCV-induced myocarditis.[52] Preliminary data from our laboratory indicate that IL-6 peaks in the heart at day 5 post-MCMV infection, whereas in the more resistant C57BL/6 mice IL-6 peaks earlier at day 3 (J. C. Lenzo, G. R. Shellam, and C. M. James, unpublished data). Therefore IL-6 stimulation appears beneficial in the acute phase of disease and deleterious in the later stages of disease.

Viral myocarditis has been treated with various forms of therapy for improved quality of life and prognosis.[53] Autoimmune reactivity has been demonstrated in most patients with myocarditis and in 30% of patients with dilated cardiomyopathy.[54–56] Our data support the theory of molecular mimicry in that suppression of anticardiac myosin antibody response correlates with reduced myocarditis. The mechanism by which type I IFNs reduce viral inflammatory heart disease include the reduction of acute virus load; and the immunomodulation of antigen-presenting cells, T cells and autoantibodies.[57,58] In this study, a reduction in acute myocarditis correlated with decreased virus load (*IFNA6*). However, at this stage additional factors must influence disease onset since *IFNA9* and *IFNB* also afforded protection against the development of acute myocarditis despite having no effect on virus load. The development of chronic myocarditis was reduced with *IFNA6* treatment. Interestingly, this was the only subtype shown to reduce virus load in all target tissues during acute infection, and in addition to decrease anticardiac myosin IgG1 autoantibodies. Previously, we observed that anticardiac myosin autoantibodies play a pathogenic role in myocarditis.[38] Alternatively, in the coxsackievirus B3 model of myocarditis, expression of IFN-γ reduced virus load with development of complete immunity and without the development of myocarditis.[59] This leads to speculation that cytokines expressed in the acute phase of infection may be effective not only at reducing virus load but in predetermining the pathogenic mechanisms leading to the development of myocarditis.

The murine and human IFNs are highly homologous. Murine *IFNA6*, at the nucleotide level, is most analagous to human *IFNA1* (74·2%), *IFNA2* (74·8%), *IFNA8* (74·4%) and *IFNA13* (74·8%), which is also reflected in amino acid alignment. Within the murine species, divergence of amino acid sequence from IFN-α6 was between 12 and 23% for the IFN-α subtypes, however, no correlation between amino acid identity and subtype effectiveness could be detected. Similarly, mutations in the IFNAR2 binding site to IFN-α (amino acids 26–34, 133, 144–153) did not correlate with antiviral activity observed *in vivo*.[60] However, IFNAR1, the second subunit of the IFN receptor, is thought to determine the signalling activity of the Type I IFNs. Mutations within the IFN-binding site of the IFNAR1 subunit may therefore be more reflective of the signal delivered to the cell and the induced immune response.

Other workers have documented long-term persistence and expression of reporter genes[61] and their potential benefits for use of this mode of DNA delivery for vaccines[62,63] and gene therapy.[64,65] Interestingly, recent research indicates that low but continuous signalling through the IFN receptor is essential for maintaining the transcription of IFN responsive genes over a longer period of time.[60] Thus, naked DNA IFN delivery has great potential as a form of cytokine therapy. Furthermore, we emphasize that the low, persistent levels of IFN protein expression, delivered via naked DNA gene therapy, may have increased therapeutic benefit over the direct inoculation of higher concentrations of IFN protein at non-physiological levels which are associated with undesirable side-effects.

In summary, our data suggest that IFN subtypes differ in their ability to alter the pathogenesis of MCMV infection. In this study we demonstrated that *IFNA6* treatment reduced virus load, enhanced production of the cytokines IFN-γ and IL-6 and reduced the abundance of anticardiac myosin autoantibodies. These results are in agreement with the proposed danger model,[66] which describes that signals delivered by the innate immune response modulate the adaptive immune response. Furthermore, the choice of IFN subtype for treatment of disease appears crucial to patient

prognosis with regard to both the acute and chronic phases of disease. This work provides support for the use of IFN gene therapy in the treatment of CMV infection and associated disease.

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