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Short communication

Co-expression of interleukin-2 to increase the efficacy of DNA vaccine-mediated protection in coxsackievirus B3-infected mice

Andreas Henke[a,*], Chi-Shiun Chiang[b], Roland Zell[a], Axel Stelzner[a]

[a] *Institute of Virology and Antiviral Therapy, Medical Center, Friedrich Schiller University, D-07740 Jena, Germany*
[b] *National Tsing Hua University, Atomic Science 101 Sec. 2, Kuang-Fu Road, Hsinchu 30013, Taiwan*

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Abstract

DNA immunizations with the major structural protein VP1 of coxsackievirus B3 (CVB3) have been previously found to protect mice from a lethal challenge with CVB3. The function of this vaccination procedure is mainly based on accelerated antibody induction with an early cytokine expression and increased virus-specific cytotoxic activity of spleen cells causing decreased myocyte destruction and reduced viral replication. Here, we report that the co-expression of the immune-stimulatory interleukin-2 (IL-2) can increase the efficacy of the inoculated DNA vaccine depending on the route of administration and the mouse strain used.

Keywords: DNA vaccine; Cytokines; Coxsackievirus

Coxsackievirus B3 (CVB3), a member of the picornavirus family and the genus enterovirus, is a commonly associated factor of acute and chronic myocarditis in humans. Every year about 5 million enterovirus infections are attributed to the coxsackieviruses group B (CVB), serotypes B1–B5 only in the United States (Kim et al., 2001). Among the wide variety of CVB-caused illnesses, those associated with myocarditis, pancreatitis, hepatitis, and transient paralysis are the more serious. Up to 12% of patients with known CVB infections may have a myocardial involvement (Gauntt et al., 1999), whereas CVB3 is the serotype most frequently associated with myocarditis (Bowles and Towbin, 1998). Despite the well characterized molecular structure of coxsackieviruses (Natarajan and Johnson, 1998) and the successful use of common vaccination strategies in animal models (Fohlman et al., 1993; See and Tilles, 1997), no virus-specific preventive procedures against CVB3 are in clinical use today. To develop such vaccination strategies, several murine models have been established (Henke et al., 1995; Mena et al., 1999). A new method to prevent virus-caused disease is the administration of plasmid DNA encoding immunogenic viral epitopes. The inoculation of DNA vaccines into muscle tissue or skin has been shown to be effective to induce humoral (Boyer et al., 1999; Konishi et al., 1999) as well as cellular (Schirmbeck et al., 1995; Qiu et al., 1999) immune responses. This technique has been effective against many pathogens, including different enteroviruses (Sin et al., 1997; Henke et al., 1998; Toniolo et al., 2002).

Using DNA plasmids encoding certain sequences of CVB3 structural proteins, it was demonstrated that after intramuscular (i.m.) inoculation the expression of the capsid protein VP1 was the most effective vaccine to protect mice against lethal CVB3 (Henke et al., 1998) or CVB4 (Toniolo et al., 2002) infections, in both experimental models, the obtained survival rate was between 70–80%. In CVB3-infected mice the application of the plasmid pCMV/VPl as a vaccine caused decreased cardiomyocyte destruction, reduced viral load in the heart tissue, accelerated antibody induction as well as activation of cytotoxic T lymphocytes (CTL) and early cytokine expression in heart tissue (Henke et al., 2001a). Therefore, the induction of B cell and/or T cell memory in vaccinated mice prior to the lethal infection could be responsible for the protection observed.

* Corresponding author. Tel.: +49 3641 657215; fax: +49 3641 657202.
E-mail address: i6hean@rz.uni-jena.de (A. Henke).

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However, the obtained protection rate by using the established DNA vaccine pCMV/VPl against CVB3 infections is still unsatisfactory. In order to increase the efficiency of this immunization procedure, several immune-stimulatory cytokines were co-expressed with the viral protein VP1. This strategy has already been successfully used to enhance the effect of DNA vaccination procedures (Operschall et al., 1999). Therefore, the coding sequences of IL-2, IL-3, IL-4, and IL-6 as well as granulocyte/macrophage-colony stimulating factor (GM-CSF) were cloned into the bicistronic expression vector pIRES (BD Biosciences, Heidelberg, Germany) which already contained the coding sequence of the viral protein VP1. The plasmid pIRES enabled the simultaneous translation of two genes of interest from the same RNA transcript. Each gene is cloned into one of the multiple cloning sites on either side of the internal ribosomal entry site (IRES) of the encephalomyocarditis virus (EMCV). The entire construct is under control of the cytomegalovirus (CMV) immediate-early promoter allowing the expression of two individual proteins from one plasmid. The following plasmids were obtained: pIRES/VPl, pIRES/VPl/IL-2, pIRES/VPl/IL-3, pIRES/VPl/IL-4, pIRES/VPl/IL-6, and pIRES/VPl/GM-CSF using the same method as described for pCMV/VPl (Henke et al., 1998). Plasmid isolation from bacteria was performed using the endotoxin-free plasmid preparation kit (Qiagen, Hilden, Germany). Expression from these plasmids was determined in vitro by transient transfection of HeLa cells. After RNA isolation, DNase digestion, and reverse transcriptase reaction, the transcriptional activity for VP1 as well as IL-2, IL-3, IL-4, IL-6 and GM-CSF of all plasmids was confirmed by PCR (data not shown). However, cytokine detection in supernatants of transfected cell cultures using enzyme-linked immunosorbent assays (ELISA) was negative.

After the expression of the DNA vaccines was analyzed in vitro, male 8–10 weeks old BALB/c or C57BL/6 mice (3–5 mice per group) were inoculated either intramuscularly (i.m.) twice in each quadriceps muscle separately with 100 μg of plasmid DNA or vaccinated via gene gun (g.g.)-mediated delivery with 1 μg plasmid DNA at 4-week intervals. One group of mice remained non-treated and one group received the parental vector pIRES as a negative control. One day prior g.g. delivery the abdominal fur of mice were removed by shaving. Murine epidermis was transfected with 1.0 μm DNA-coated gold microcarriers by holding the plastic spacer of the Helios gene gun (Bio-Rad Laboratories, Munich, Germany) directly against the target site. A helium pressure of 400 psi was applied. Four weeks after each immunization, sera were analyzed for the presence of CVB3-specific antibodies by ELISA, using purified CVB3 as a target antigen (Henke et al., 2001b). All sera obtained prior to immunization were negative for CVB3 antibodies. In sera of mice of both strains no increase of virus-specific antibodies was detectable after the first vaccine inoculation. However, four weeks after the second immunization slightly enhanced antibody concentrations were found in all sera of mice which were treated with plasmid DNA encoding the VP1 sequence of CVB3. The highest level was detectable in pIRES/VPl/IL-2-vaccinated BALB/c mice after i.m. inoculation and in pIRES/VPl/IL-2-vaccinated C57BL/6 mice after gene gun application (data not shown). Due to the fact, that enhanced antibody levels in DNA-vaccinated mice were most likely responsible for the induced protection (Henke et al., 2001a), the following experiments were carried out using only this plasmid DNA for vaccination with both murine strains.

Four weeks after the second immunization, all mice were subjected to intraperitoneal (i.p.) challenge with five LD_{50}'s of the lethal CVB3H3 virus variant (Knowlton et al., 1996). After this challenge, the number of surviving animals was monitored up to 4 weeks post-infection (p.i.). As it is shown in Fig. 1, two i.m. inoculations of pIRES/VPl/IL-2 protected 85% (17 out of the 20) BALB/c mice against the lethal CVB3H3 challenge whereas all other vaccinated mice were less protected. The plasmid pIRES/VPl with-

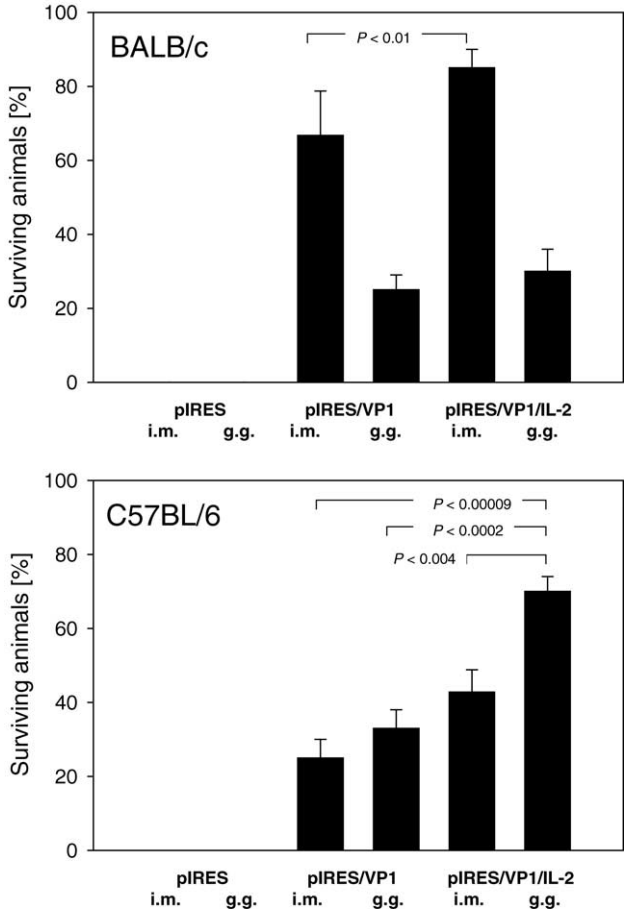

Fig. 1. DNA vaccine-induced protection against lethal CVB3 challenge. BALB/c or C57BL/6 mice were non-treated (control) or intramuscularly (i.m.)- or gene gun (g.g.)-inoculated with the control plasmid pIRES or with plasmid DNA encoding either VP1 of CVB3 alone (pIRES/VPl), or VP1 and IL-2 sequences (pIRES/VPl/IL-2). Four weeks after the second inoculation, mice were challenged with five LD_{50} doses of CVB3H3 i.p. The percentage of surviving animals is shown monitored over a time period of 28 days. The results presented are summarized data of three independent experiments using three to five mice in each group. Significant differences are indicated.

out IL-2 co-expression induced 66.7% (8 out of the 12) protection, thus confirming the results we obtained before with the pCMV/VPl vaccine: 72.2% protection (Henke et al., 1998) or 71.4% (Henke et al., 2001a). The statistical analysis (Microsoft Excel by using Student's *t*-test) demonstrates significant differences between pIRES/VPl/IL-2- and pIRES/VPl-vaccinated mice ($P < 0.01$). Gene gun-inoculations of pIRES/VPl or pIRES/VPl/IL-2 were less effective as they induced incomplete protection of 25% (3 of the 12 with pIRES/VPl) or 30% (3 of the 10 with pIRES/VPl/IL-2). The use of pIRES without VP1 or IL-2 caused no protection at all. Animals of the genetic different mouse strain C57BL/6 were better protected against CVB3H3 after vaccination with the g.g. method. As shown in Fig. 1, two g.g. inoculations of pIRES/VPl/IL-2 protected 70% (7 out of the 10) C57BL/6 mice against the lethal CVB3H3 challenge, whereas all other vaccinated mice were less protected. The g.g. injection of the plasmid pIRES/VPl without IL-2 co-expression induced 33.3% (three out of the nine) protection. Intramuscular inoculation of pIRES/VPl or pIRES/VPl/IL-2 was less effective, inducing incomplete protection of 25% (3 of the 12 with pIRES/VPl) or 42.8% (6 of the 14 with pIRES/VPl/IL-2). The statistical analysis revealed significant differences between pIRES/VPl/IL-2- and pIRES/VPl-inoculated mice after g.g. treatment ($P < 0.0002$), between pIRES/VPl/IL-2 g.g.- and pIRES/VPl/IL-2 i.m.-treated mice ($P < 0.004$), as well as pIRES/VPl/IL-2 g.g.- and pIRES/VPl i.m.-vaccinated mice ($P < 0.00009$). The use of pIRES without VP1 or IL-2 caused no protection.

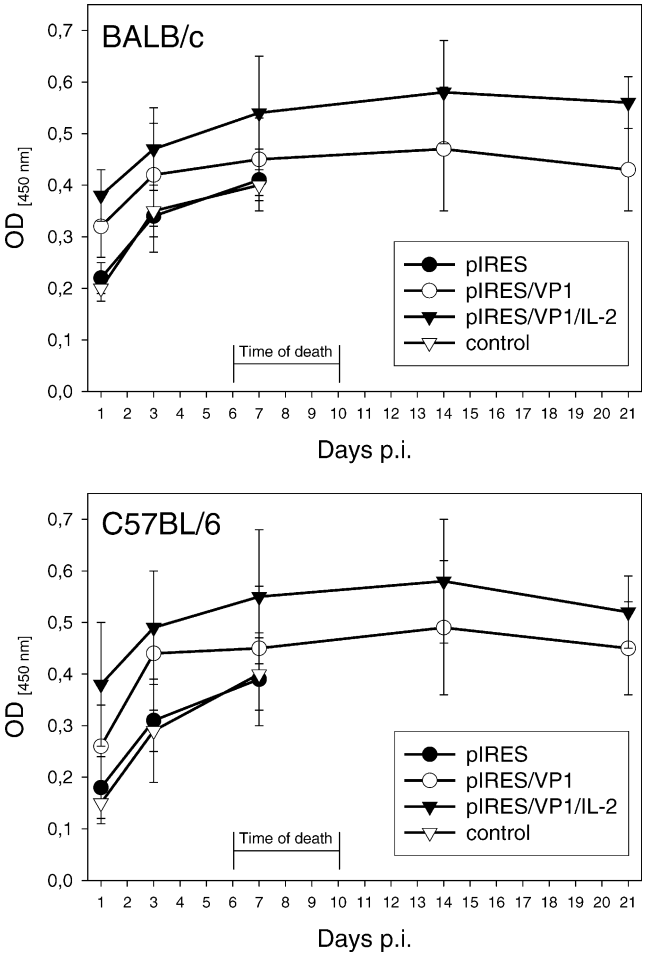

Fig. 2. Characterization of the antibody response after a lethal CVB3H3 challenge. BALB/c mice were non-treated (control) or i.m.- and C57BL/6 were non-treated (control) or g.g.-inoculated with the control plasmid pIRES or with plasmid DNA encoding either VP1 of CVB3 alone (pIRES/VPl), or VP1 and IL-2 sequences (pIRES/VPl/IL-2). Four weeks after the second inoculation, mice were challenged with five LD_{50} doses of CVB3H3 i.p. The amount of CVB3-specific antibodies—detected by ELISA (serum dilution 1:25) and demonstrated as the optical density at 490 nm (OD_{490})—was analyzed in sera of individual mice during 3 weeks p.i. Non-protected mice died between 6 and 10 days p.i. Four mice in each group were used in each experiment. The data shown are the mean values ± standard deviation. The results are representative of three different experiments.

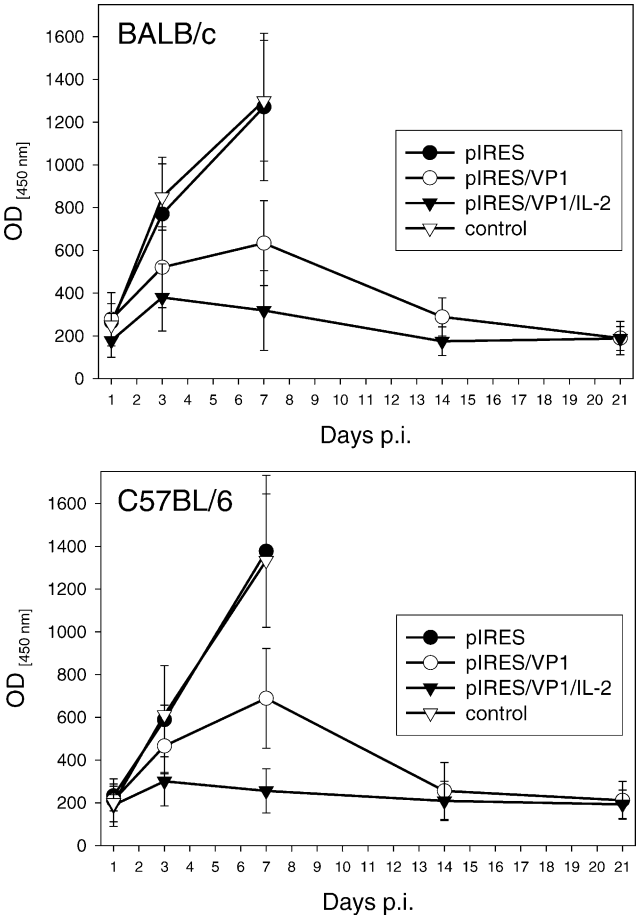

Fig. 3. Characterization of the cardiocvte-specific enzyme α-HBDH in murine sera after the lethal CVB3H3 challenge. BALB/c mice were non-treated (control) or i.m.- and C57BL/6 were non-treated (control) or g.g.-inoculated with the control plasmid pIRES or with plasmid DNA encoding either VP1 of CVB3 alone (pIRES/VPl), or VP1 and IL-2 sequences (pIRES/VPl/IL-2). Four weeks after the second inoculation, mice were challenged with five times the LD_{50} of CVB3H3 i.p. The concentration of the cardiocyte-specific enzyme α-HBDH was analyzed in sera of individual mice during 3 weeks p.i. Non-protected mice died between 6 and 10 days p.i. Four mice in each group were used in each experiment. The data shown are the mean values ± standard deviation. The results are representative of three different experiments.

To analyze if this vaccination procedure could reduce the virus-induced pathological changes in mice, the following approach was used: male BALB/c or C57BL/6 mice were inoculated with pIRES or pIRES/VPl as controls or with pIRES/VPl/IL-2 via the i.m. or the g.g. route depending on the mouse strain used. At the indicated days after a lethal CVB3H3 challenge with five LD_{50}, mice were sacrificed and analyzed for the presence of virus-specific antibodies in sera (Fig. 2), viral load in pancreas and heart tissue (Table 1) as well as serum concentration of the cardiomyocyte-specific enzyme α-hydroxybutyrate dehydrogenase (α-HBDH), using a commercially available kit (Sigma Diagnostics, St. Louis, MO) according to the instructions of the manufacturer (Fig. 3) and described previously (Henke et al., 2001b). The time of death in all groups occurred between 6–10 days p.i.

The vaccination procedure induced low levels of anti-CVB3 antibodies. But early after the challenge, increased concentrations of CVB3-specific antibodies were detectable in sera of pIRES/VPl/IL-2-vaccinated mice, indicating that the vaccination procedure induced an immunologic memory which was accompanied by a secondary antibody response. During the observation time (1–21 days post-challenge) the antibody titer in sera of the pIRES/VPl/IL-2-vaccinated groups remained always higher than in the control groups, independently from the inoculation route and the mouse strain used (Fig. 2). This higher antibody titer in sera of the pIRES/VPl/IL-2-vaccinated mice was accompanied by a reduced viral load, indicating that these antibodies lead to decreased viral replication in murine tissue. Infectious virus was isolated from pancreas and heart tissue of the same mice as it was described previously (Henke et al., 1995). The virus titer was determined by cell culture infectious dose 50% assays ($CCID_{50}$) on HeLa cell monolayers (Table 1). In both pIRES/VPl/IL-2-treated BALB/c and C57BL/6 mice the viral replication in pancreas and heart tissue was reduced and delayed during the observation time in comparison with both controls. At 21 days p.i. all surviving animals were able to stop the viral replication. As demonstrated in Fig. 3, high levels of α-HBDH-activity were detectable in sera of pIRES-treated control mice starting 3 days after the challenge. In contrast, the α-HBDH activity in sera of pERES/VPl- and especially pIRES/VPl/IL-2-vaccinated mice was decreased. The reduced viral replication in the heart tissue caused significant less damage in cardiomyocytes of vaccinated mice. Heart tissue of surviving animals did not show histopathological changes, indicating that CVB3H3 was unable to induce tissue destruction, massive inflammation, or fibrosis 50 days p.i. (data not shown).

The nature of the plasmid DNA-induced immune response is influenced by delivery routes and methods of delivery. Intramuscular inoculation drives immune responses mainly towards the Thl-response (Raz et al., 1996). The g.g. delivery method requires much less DNA as compared to the i.m. method and drives immune responses to both the Thl- (Feltquate et al., 1997) and the Th2-type (Prayaga et al., 1997). The mechanism of this selective Thl- and Th2-specific immune activation by different DNA inoculation methods is presently not well understood. The co-injection of the IL-2 gene during DNA immunization studies enhanced the development of Thl cells, while the Th2 activation was not affected (Chow et al., 1998). In addition, the genetically differ-

Table 1
Virus concentration in pancreas and heart tissue of BALB/c and C57BL/6 mice during the challenge period [log $CCID_{50}$/0.1 g tissue][a]

Plasmid	Organ	Days (post-challenge)				
		1	3	7	14	21
BALB/c						
pIRES	Pancreas	7.32 ± 4.78	8.33 ± 6.21	6.12 ± 4.87	–[b]	–
	Heart	3.48 ± 2.88	6.35 ± 4.89	6.75 ± 5.67	–	–
pIRES/VP1	Pancreas	6.25 ± 5.66	7.67 ± 6.21	5.52 ± 5.01	n.d.[c]	n.d.
	Heart	3.52 ± 2.34	5.22 ± 3.89	4.66 ± 4.78	2.5 ± 2.23	n.d.
pIRES/VP1/IL-2	Pancreas	5.33 ± 4.07	4.97 ± 3.89	2.31 ± 1.98	n.d.	n.d.
	Heart	n.d.	3.21 ± 3.01	3.45 ± 2.33	n.d.	n.d.
C57BL/6						
pIRES	Pancreas	7.01 ± 5.67	8.88 ± 6.73	6.65 ± 5.12	–	–
	Heart	3.55 ± 2.29	6.05 ± 4.67	6.67 ± 5.11	–	–
pIRES/VP1	Pancreas	6.34 ± 5.25	7.56 ± 6.77	5.89 ± 4.98	n.d.	n.d.
	Heart	4.12 ± 3.67	5.13 ± 4.23	4.77 ± 4.09	n.d.	n.d.
pIRES/VP1/IL-2	Pancreas	5.89 ± 4.73	4.45 ± 4.02	2.87 ± 2.23	n.d.	n.d.
	Heart	n.d.	3.89 ± 2.89	3.32 ± 2.67	n.d.	n.d.

[a] BALB/c mice were i.m.- and C57BL/6 were g.g.-inoculated with the control plasmid pIRES or with plasmid DNA encoding either VP1 of CVB3 alone (pIRES/VPl), or VP1 and IL-2 sequences (pIRES/VPl/IL-2). Four weeks after the second inoculation, mice were challenged with five LD_{50} doses of CVB3H3 i.p. Four mice in each group were used in each experiment. The data shown are the mean values ± standard deviation. The results are representative of three different experiments.

[b] No surviving animals.

[c] Not detectable.

ent BALB/c and C57BL/6 mice responded in certain models of infectious disease either more towards the Thl or the Th2 pathway (Sabin and Pearce, 1995; Kondratieva et al., 2000). Therefore, the efficacy of DNA immunization procedures and the induced immune reactions are influenced by several different factors like, e.g., the individual response to the vaccine and the infectious agent, the route of vaccine delivery, and the application of immune-stimulatory cytokines.

In conclusion, the inoculation of the bicistronic plasmid DNA pIRES/VPl/IL-2 induced the highest virus-specific antibody concentrations in sera of BALB/c after i.m. injection or in C57BL/6 mice after g.g. inoculation in comparison to the application of other plasmids like pIRES/VPl, pIRES/VPl/IL-3, pIRES/VPl/IL-4, pIRES/VPl/IL-6, and pIRES/VPl/GM-CSF. Therefore, pIRES/VPl/IL-2 was used for further protection experiments. After i.m. administration, IL-2 co-expression increased the protection rate by 18.3% whereby the g.g. inoculation was much less effective in BALB/c mice. In contrast, after g.g. inoculation IL-2 co-expression increased the protection rate by 36.7% whereby the i.m. was much less effective in C57BL/6 mice. Following experiments were focused on the characterization of the vaccine-mediated protection. During 3 weeks after the lethal challenge i.m., pIRES/VPl/IL-2-vaccinated BALB/c mice as well as g.g. pIRES/VPl/IL-2-vaccinated C57BL/6 mice revealed higher virus-specific serum antibody concentrations, reduced viral replication in pancreas and heart and less myocardial damage, indicating induced immunologic memory in vaccinated mice prior challenge in comparison to control mice. Future experiments will be focused on the characterization of the induced immune response with regard to the induction of Thl- versus Th2-specific reactions and a further increase of the efficiency of the established DNA vaccine pIRES/VPl/IL-2.

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References

Bowles, N.E., Towbin, J.A., 1998. Molecular aspects of myocarditis. Curr. Opin. Cardiol. 13, 179–184.

Boyer, J.D., Kim, J., Ugen, K., Cohen, A.D., Ahn, L., Schumann, K., Lacy, K., Bagarazzi, M.L., Javadian, A., Ciccarelli, R.B., Ginsberg, R.S., MacGregor, R.R., Weiner, D.B., 1999. HIV-1 DNA vaccines and chemokines. Vaccine 17 (Suppl. 2), S53–S64.

Chow, Y.H., Chiang, B.L., Lee, Y.L., Chi, W.K., Lin, W.C., Chen, Y.T., Tao, M.H., 1998. Development of Thl and Th2 populations and the nature of immune responses to hepatitis B virus DNA vaccine can be modulated by codelivery of various cytokine genes. J. Immunol. 160, 1320–1329.

Feltquate, D.M., Heaney, S., Webster, R.G., Robinson, H.L., 1997. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. J. Immunol. 158, 2278–2284.

Fohlman, J., Pauksen, K., Morein, B., Bjare, U., Ilback, N.G., Friman, G., 1993. High yield production of an inactivated coxsackie B3 adjuvant vaccine with protective effect against experimental myocarditis. Scand. J. Infect. Dis. 88 (Suppl.), 103–108.

Gauntt, C.J., Sakkinen, P., Rose, N.R., Huber, S.A., 1999. Picornaviruses: immunopathology and autoimmunity. In: Cunningham, M.W., Fujinami, R.S. (Eds.), Effects of Microbes on the Immune System. Lippincott-Raven Publishers, Philadelphia, pp. 313–329.

Henke, A., Huber, S., Stelzner, A., Whitton, J.L., 1995. The role of CD8+ T lymphocytes in coxsackievirus B3-induced myocarditis. J. Virol. 69, 6720–6728.

Henke, A., Wagner, E., Whitton, J.L., Zell, R., Stelzner, A., 1998. Protection of mice against lethal coxsackievirus B3 infection by using DNA immunization. J. Virol. 72, 8327–8331.

Henke, A., Zell, R., Stelzner, A., 2001a. DNA vaccine-mediated immune responses in coxsackievirus B3-infected mice. Antiviral Res. 49, 49–54.

Henke, A., Zell, R., Ehrlich, G., Stelzner, A., 2001b. Expression of immunoregulatory cytokines by recombinant coxsackievirus B3 variants confers protection against virus-caused myocarditis. J. Virol. 75, 8187–8194.

Kim, K.S., Hufhagel, G., Chapman, N.M., Tracy, S., 2001. The group B coxsackieviruses and myocarditis. Rev. Med. Virol. 11, 355–368.

Knowlton, K.U., Jeon, E.S., Berkley, N., Wessely, R., Huber, S.A., 1996. A mutation in the puff region of VP2 attenuates the myocarditic phenotype of an infectious cDNA of the Woodruff variant of coxsackievirus B3. J. Virol. 70, 7811–7818.

Kondratieva, T.K., Kobets, N.V., Khaidukov, S.V., Yeremeev, V.V., Lyadova, I.V., Apt, A.S., Tarn, M.F., Stevenson, M.M., 2000. Chraracterization of T cell clones derived from lymph nodes and lungs of *Pseudomonas aeruginosa*-susceptible and resistant mice following immunization with heat-killed bacteria. Clin. Exp. Immunol. 121, 275–282.

Konishi, E., Yamaoka, M., Khin Sane, W., Kurane, L., Takada, K., Mason, P.W., 1999. The anamnestic neutralizing antibody response is critical for protection of mice from challenge following vaccination with a plasmid encoding the Japanese encephalitis virus premembrane and envelope genes. J. Virol. 73, 5527–5534.

Mena, I., Perry, C.M., Harkins, S., Rodriguez, F., Gebhard, J., Whitton, J.L., 1999. The role of B lymphocytes in coxsackievirus B3 infection. Am. J. Pathol. 155, 1205–1215.

Natarajan, P., Johnson, J.E., 1998. Molecular packing in virus crystals: geometry, chemistry, and biology. J. Struct. Biol. 121, 295–305.

Operschall, E., Schuh, T., Heinzerling, L., Pavlovic, J., Moelling, K., 1999. Enhanced protection against viral infection by co-administration of plasmid DNA coding for viral antigen and cytokines in mice. J. Clin. Virol. 13, 17–27.

Prayaga, S.K., Ford, M.J., Haynes, J.R., 1997. Manipulation of HIV-1 gp 120-specific immune responses elicited via gene gun-based DNA immunization. Vaccine 15, 1349–1352.

Qiu, J.T., Song, R., Dettenhofer, M., Tian, C., August, T., Felber, B.K., Pavlakis, G.N., Yu, X.F., 1999. Evaluation of novel human immunodeficiency virus type 1 gag DNA vaccines for protein expression in mammalian cells and induction of immune responses. J. Virol. 73, 9145–9152.

Raz, E., Tighe, H., Sato, Y., Corr, M., Dudler, J.A., Roman, M., Swain, S.L., Spiegelberg, H.L., Carson, D.A., 1996. Preferential induction of a Thl immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. Proc. Natl. Acad. Sci. USA 93, 5141–5145.

Sabin, E.A., Pearce, E.J., 1995. Early IL-4 production by non-CD4+ cells at the site of antigen deposition predicts the development of a T helper 2 cell response to *Schistosoma mansoni* eggs. J. Immunol. 155, 4844–4853.

Schirmbeck, R., Bohm, W., Ando, K., Chisari, F.V., Reimann, J., 1995. Nucleic acid vaccination primes hepatitis B virus surface antigen-specific cytotoxic T lymphocytes in nonresponder mice. J. Virol. 69, 5929–5934.

See, D.M., Tilles, J.G., 1997. Occurrence of coxsackievirus hepatitis in baby rabbits and protection by a formalin-inactivated polyvalent vaccine. Proc. Soc. Exp. Biol. Med. 216, 52–56.

Sin, J.I., Sung, J.H., Suh, Y.S., Lee, A.H., Chung, J.H., Sung, Y.C., 1997. Protective immunity against heterologous challenge with encephalomyocarditis virus by VP1 DNA vaccination: effect of coinjection with a granulocyte-macrophage colony stimulating factor gene. Vaccine 15, 1827–1833.

Toniolo, A., Falcone, V., Bemasconi, C., Basolo, F., Speziale, P., Onodera, T., 2002. DNA immunization of mice against the VP1 capsid protein of coxsackievirus B4. Scand. J. Immunol. 56, 448–455.