## S2-4

# Pathogenic Roles of the Viral Immunoreceptor Tyrosine Activation Motif of the Coxsackievirus VP2 Influencing NF-κB Modulated Cytokine Induction: Its Application as a Novel Viral Vector System and Vaccine for Enteroviruses

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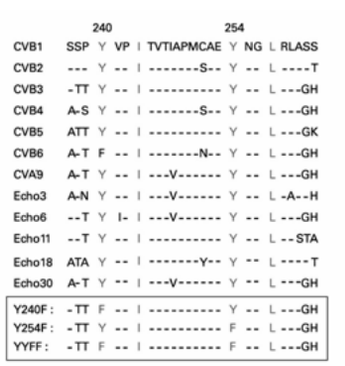
The immunoreceptor tyrosine-based activation motif (ITAM) [Yxx(L/I)x6-12Yxx(L/I)] sequences conserved in B-cell and T-cell receptors are necessary for the coupling of extracellular signals to intracellular signaling molecules, which result in cellular activation and proliferation. Interestingly, it has been known that some viral proteins encode ITAM sequences which may affect viral pathogenesis. However, the detailed mechanisms of its pathogenic effect remains poorly understood. This study revolves around the identification of an ITAM sequences in the C-terminal of enetroviruses, including coxsackieviruses, capsid protein VP2 (Fig. 1). The mutant viruses with phenylalanines substituted in two tyrosines in ITAM appear to be highly attenuated, and achieves such ends as the reduction of the mortality rate and of viral titers, as well as the down-regulation of proinflammatory cytokines in infected mice (Fig. 2). Interestingly, the wild type virus (WT) could induce nuclear factor kappa B (NF-κB) activation, in contrast to YYFF, which did not in macrophage cells. Moreover, YYFF VP2 partially hindered NF-κB activation by the agonist (Fig. 3). The data proves that some of the cytokine signaling cascades of the mutant viruses have been impeded, and that they have defective signaling pathways.



| | 240 | | | | | | 254 | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| CVB1 | SSP | Y | VP | I | TVTIAPMCAE | Y | NG | L | RLASS |
| CVB2 | --- | Y | -- | I | -------S-- | Y | -- | L | ----T |
| CVB3 | -TT | Y | -- | I | ---------- | Y | -- | L | ---GH |
| CVB4 | A-S | Y | -- | I | -------S-- | Y | -- | L | ---GH |
| CVB5 | ATT | Y | -- | I | ---------- | Y | -- | L | ---GK |
| CVB6 | A-T | F | -- | I | -------N-- | Y | -- | L | ---GH |
| CVA9 | A-T | Y | -- | I | ---V------ | Y | -- | L | ---GH |
| Echo3 | A-N | Y | -- | I | ---V------ | Y | -- | L | -A--H |
| Echo6 | --T | Y | I- | I | ---V------ | Y | -- | L | ---GH |
| Echo11 | --T | Y | -- | I | ---------- | Y | -- | L | --STA |
| Echo18 | ATA | Y | -- | I | -------Y-- | Y | -- | L | ----T |
| Echo30 | A-T | Y | -- | I | ---V------ | Y | -- | L | ---GH |
| Y240F : | -TT | F | -- | I | ---------- | Y | -- | L | ---GH |
| Y254F : | -TT | Y | -- | I | ---------- | F | -- | L | ---GH |
| YYFF : | -TT | F | -- | I | ---------- | F | -- | L | ---GH |

Fig. 1. The amino acid alignment of the ITAM sequences in C-terminal of VP2 protein of enteroviruses

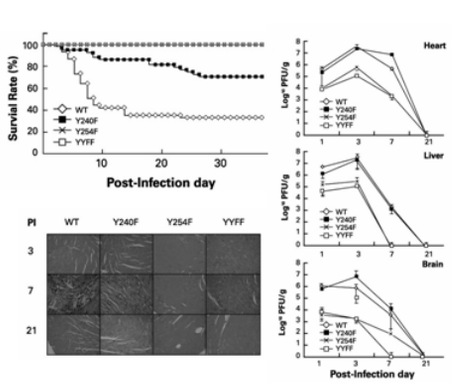



Fig. 2. Survival rate, viral titrt in mouse organ, and histology of myocardil inflammation

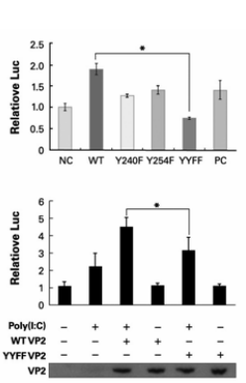



Fig. 3. NF-κB activation by viruses in macrophage cells and inhibition of NF- κB activity by YYFF VP2

Ultimately, mutant viruses were unable to induce the proinflammatory cytokines triggering the tissue injury via NF-κB activation. Taken together, this constitutes the first known revelation of the presence of an ITAM sequence in coxsackievirus, and provides direct evidence that viral ITAM affects viral pathogenesis via NF-κB regulated inflammatory cytokine induction.

For the application of the above mutant virus, YYFF, we evaluated the possibility whether YYFF could be used as a live vaccine for group B coxsackieviruses. After having been immunized through intraperitoneal (ip) and subcutaneous (sc) routes, YYFF was found to induce a neutralizing antibody against CVB3 with both routes inducing similar neutralizingantibody titers (1:640 at 4 weeks post-immunization, pi, and 1:160 at 10 weeks pi). The neutralizing antibodies were maintained at least 30 weeks pi. Following immunization, the viral titer was found to reach its peak in the heart, liver, and pancreas at 3 days pi, before decreasing thereafter. YYFF immunization did not induce inflammation in the heart, or proinflammatory cytokines such as IL-6 and TNF-α. In order to verify the efficacy of YYFF, four-week-old BALB/c mice were immunized intraperitoneally with $10^6$ plaque forming units (PFU) of YYFF, and challenged with $10^6$ PFU of CVB 1-6 at day 14 post-infection. In terms of survival rate, YYFF demonstrated a perfect protection effect against the CVB3 challenge (100% survival rate). Furthermore, the viral titers decreased 2-4 fold in log scale in the mice organs following the CVB3 challenge. As expected, YYFF immunization resulted in protecting the heart and pancreas from inflammation caused by the CVB3 challenge. Surprisingly, YYFF also guarded against inflammation in the pancreas emanating from the challenge of other group B coxsackieviruses (CVB1 to CVB6), while also reducing viral titers in the heart, liver and pancreas (Fig. 4). Taken together, the above data suggests that YYFF may serve as a potential live vaccine candidate for not only CVB3, but other group B coxsackieviruses as well.

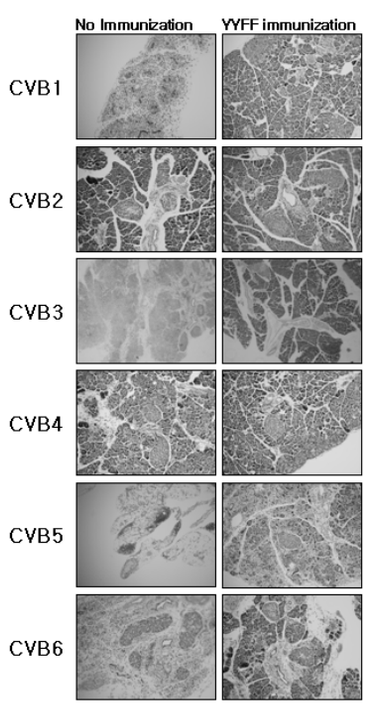


Fig. 4. Protection of pancreatitis by YYFF immunization

We also explored the possibility of YYFF as a recombinant viral vector that could be used to deliver foreign genes into mice for the purpose of vaccination. We constructed recombinant YYFF cDNA by inserting a truncated form of the gene encoding hepatitis C virus envelope protein E2 (384-661 amino acids of HCV genome) immediately upstream from the VP0 capsid protein of the CVB3. The infectious virus (YYFF-HCV) was raised in HeLa cells to titers of $6\times10^6$ PFU/ml. However, the titer of the YYFF-HCV chimeric virus was 1-2 fold lower in log scale than that of the YYFF virus.

Using Western blot and immunofluorescence assays with anti-HVR1 rabbit hyperimmune sera (LMF86), it was discovered that the YYFF-HCV virus was capable of expressing the truncated HCV E2 protein in various cells such as HeLa (epithelial cells), Jurkat (T cells), Raji (B cells) and RAW264.7 (Macrophage cells)(Fig. 5). The HCV E2 protein expression was maintained after at least five passages in the tested cells. In order to evaluate the immune response *in vivo*, one group of 8-week-old male Balb/c mice were intraperitoneally infected with $10^6$ pfu of YYFF-HCV, and another group, used as a positive control, were immunized through an intramuscular injection of pE2surf-661 plasmid containing the truncated HCV E2 gene (384-661 amino acids of the HCV genome). Although both groups of immunized mice showed anti-HCV antibodies, which was detected using Western blot and immunofluorescence assays, the YYFF-HCV infection induced a higher degree of antibody titer than was the case with the pE2surf-661 injection (Fig. 6). These results demonstrate that the chimeric CVB3 expressing foreign gene could serve as a live vaccine capable of eliciting antibodies directed against a foreign antigenic epitope.

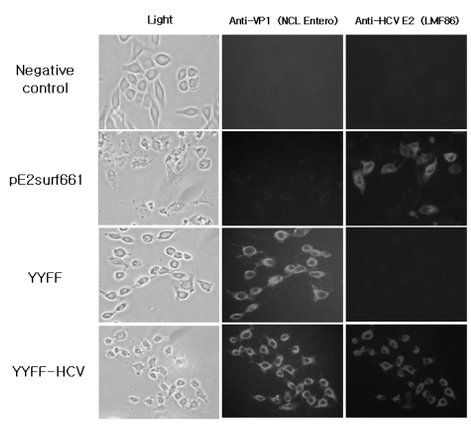



Fig. 5. Immunofluorescence assay for detection of the truncated HCV E2 protein from YYFF-HCV in HeLa cells

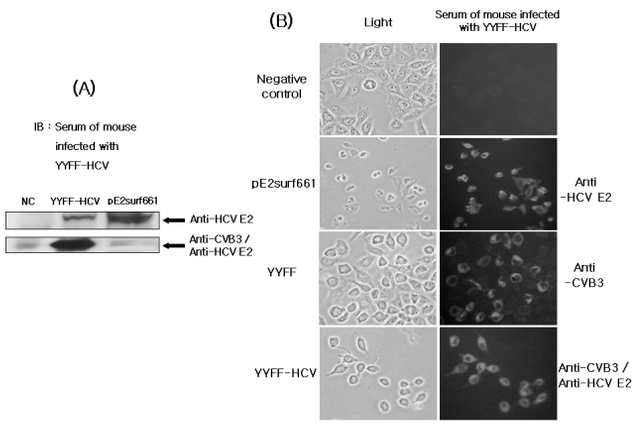



Fig. 6. Production of anti-HCV E2 in mouse immunized with YYFF-HCV