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Anti-IL-22 neutralizing antibodies decrease inflammation lesions and reduce mortality in enterovirus 71-infected mice

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ABSTRACT

Hand, foot, and mouth disease (HFMD) can cause fatal encephalitis in 0-5-year-old infants and children. There is no effective antiviral drug available to treat HFMD caused by enterovirus 71 (EV71). Our study investigates the relationship between levels of IL-22 expression and the severity of disease after EV71 infection in a mouse model. Anti-IL-22 neutralizing antibodies were tested in EV71-infected mice of different ages. Our results show that anti-IL-22 neutralizing antibodies can effectively reduce mortality in EV71-infected mice. Anti-IL-22 neutralizing antibody effectively reduced various EV71-associated symptoms indicating promising potential of this therapeutic effector in patients with EV71-associated HFMD.

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Introduction

Hand, foot, and mouth disease (HFMD) can cause fatal encephalitis in 0-5-year-old infants and children. The disease is initially self-limited, but in severe cases, it can be followed by severe neurological symptoms that can be fatal. Severe HFMD patients indicated long-term neurological sequelae, delayed neurodevelopment, and reduced cognitive functioning. It has been shown that 90% of the severe cases are caused by enterovirus 71 (EV71), which is specifically prevalent in Asia-Pacific regions with economically underdeveloped areas and poor public health management. HFMD outbreaks are marked by a certain periodicity, with a large epidemic occurring every 2-3 years (1). The largest recent EV71 outbreak occurred in Fuyang, Anhui, China, in 2008. A total of 490,000 infection cases, including 126 deaths, were reported during the outbreak (2). In a previous EV71 epidemic in Bulgaria (1975), the clinical HFMD manifestations were mostly recognized as adverse central nervous system (CNS) symptoms. In this outbreak, hundreds of cases were reported, including 545 cases (77.3%) of aseptic meningitis and 149 cases (21.1%) of acute flaccid paralysis. Among all of these cases, 44 deaths (6.2%) were reported, almost all of which were caused by bulbar injury (3). Notably, there is no effective antiviral drug available to treat HFMD caused by EV71 or similar viral infections (4). Only supportive care and intravenous antibodies are available as a treatment for severe cases (5). However, the antibodies currently used to treat EV71 infection were generated against the virus itself, and the therapeutic efficacy of this treatment seems limited (6). The vaccine against EV71 has demonstrated preventive effects against the disease in clinical trials, but the vaccine is useless in patients who have already developed the disease (6). The deterioration process is very rapid in severe HFMD cases. Currently, there are no effective methods or indicators for early identification and diagnosis of severe HFMD disease development.

Besides CNS-linked symptoms, EV71 infection was found to trigger strong pro-inflammatory responses associated with systemic immune system failure in humans (1,5). This viral infection has been described as a direct T cell receptor (TCR) signaling profile (10). Activated TCR cascade is associated with the release of various regulatory effectors, including common γ-chain cytokines. For instance, an animal model of EV71 infection indicated both cellular and humoral immune responses, including elevated levels of Th17, Th1, Th2, and CD8+T cells (12). A distinct subset of the Th cell family, which can produce IL-22 (Th22 cells), was linked to abnormally high immune responses in multiple diseases, including skin infections (13). Recently, IL-22 anti-viral effect was shown to abrogate respiratory syncytial virus-induced respiratory pathologies (14) indicating on potential importance of this cytokine in the resolution of viral infections.

IL-22 is a member of the IL-10 family or IL-10 superfamily, a class of potent mediators of cellular inflammatory responses (other members include IL-19, IL-20, IL-24, and IL-26) (7). The targets of this cytokine are mostly non-hematopoietic cells. IL-22 activates intracellular kinases (JAK1, Tyk2, and MAP kinases) and transcription factors, especially STAT3 (8). Studies have shown that IL-22 plays an important role in a variety of cancerous, chronic inflammatory, and infectious diseases. Recently, it has been shown that frequencies of interleukin IL-22+CD4+T (Th22) cells were significantly elevated in severely affected HFMD patients (13). However, the role of the IL-22 blockade as an antiviral drug treatment in severe cases of EV71 infection has not yet been tested. In this study, we used anti-IL-22 neutralizing antibodies and investiga-

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ted the relationship between IL-22 and disease severity in a mouse model of EV71 infection *in vivo*. Our results showed that the level of IL-22 expression was positively correlated with disease severity and that anti-IL-22 neutralizing antibody effectively reduced mortality in EV71-infected mice by decreasing inflammatory lesions.

Materials and Methods

Cell and virus

The human muscular (rhabdomyosarcoma (RD)) cell line was maintained in a medium according to American Type Culture Collection instructions. EV71 strain GD 10-12 (GenBank accession number KJ004559) was isolated from a patient in Guangdong province, in 2010. Then it was passed 5 times in mice to obtain MP5, the mouse-adapted strain. MP5 was then propagated and titrated in RD cells, and used to infect mice.

Animal model

7-21-day-old BALB/c mice were infected with 3×10^8 pfu per gram body weight of MP5 using intraperitoneal inoculation. Following this, 24 h after infection, the mice were given 1 intraperitoneal injection of PBS (controls) or anti-IL-22 neutralizing antibody (20 mg, daily) for 5 days. Infected mice were monitored for signs of disease and survival for 10 days. Mice clinical scores were graded as follows: 0, healthy; 1, ruffled hair; 2, weakness in hind limbs; 3, paralysis in single hind limbs; 4, paralysis in both hind limbs; and 5, death. To assess IL-22 level and the tissue damage, infected mice were euthanized and mice hindlimb muscles were collected on day 3 after infection. IL-22 tissue level was tested by ELISA, while the tissue damage was evaluated using histopathological routine H&E staining. Anti-IL-22 neutralizing antibody experiments were performed on 7-14 days-old mice.

Statistical analysis

Difference of the mortality, body weight and subsequent paralysis sequelae of 7,14,18 and 21-day-old EV71- infected mice were evaluated by mean SE values. P evaluated by log-rank test (survival rates), Wilcoxon test (clinical scores), and Mann-Whitney U test (relative change of IL-22). P values < 0.05 were considered statistically significant.

Results

The severity of disease after infection was related to the age of the mice

The experimental infection was tested in mice of different ages. We found that younger mice infected with EV71 developed more serious disease. Accordingly, 7, 14 and 18-day-old mice can survive up to 3, 4, and 7 days after EV71 infection, respectively. Notably, the virus did not cause death in 21-day-old mice (Figure 1A). All mice stopped gaining weight after infection and began to lose weight on the third day after infection, except for the 21-day-old mice (Figure 1B). Different age mice showed clinical symptoms at 1, 3, and 4 days after infection (Figure 1C); however, 21-day-old mice did not develop paralysis in their hind limbs.

To investigate factors that can correlate to the severity of the disease, the transcriptional level of genes was compared before and after the introduction of EV71 infection in mice. According to the results of gene expression analysis, we tested the level of cytokines that indicated changes during the genetic analysis. We found that IL-22 was differentially expressed in different-age mice after EV71 infection (Figure 1D). The severity of the disease decreased in older mice. Simultaneously, IL-22 expression level also decreased gradually in the muscles of older mice. Therefore, we speculated that IL-22 is positively correlated with the severity of EV71-induced disease. We suggest that IL-22 plays an important role in HFMD development and complications.

Antibodies can effectively reduce inflammation after infection

To test our hypothesis, we treated EV71-infected 14-day-old mice with anti-IL-22 neutralizing antibodies. The results of this treatment showed that anti-IL-22 neutralizing antibodies effectively reduced the mortality of the infected mice. The anti-IL-22 antibody-treated mice showed a marked reduction in mortality with a survival rate of 80% (8/10) which is significantly higher than that in PBS-treated mice ($P < 0.05$, log-rank test) (Figure 2A). Alternatively, all infected control mice developed hind limb paralysis with signs of encephalitis manifested by hunched posture, lethargy, ataxia, and anorexia. None of the control group mice (0%; 0/10) survived.Anti-IL-22 antibody-treated mice did not show obvious pro-inflammatory symptoms on day 3 after infection, whereas PBS-treated control mice began to develop paralysis in their hind limbs. H&E staining confirms the higher level of inflammation in control mice tissue.

After EV71 injection, PBS-treated control mice experienced sustained weight loss until their death. Alternatively, IL-22 antibody-treated mice gained weight in the first 3 days after infection. Following this, the antibody-treated mice lost weight from the 4th to 8th day but regained weight on the 9th day (Figure 2B). Accordingly, EV71-related clinical symptoms were almost gone on day 9th in the IL-22 antibody-treated mice (Figure 2C). The mortality rate of the antibody-treated mice was 0% until

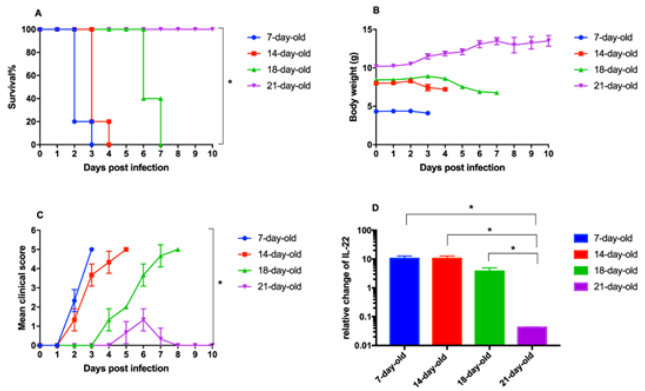

Figure 1. Difference of the mortality, body weight and subsequent paralysis sequelae of 7,14,18 and 21-day-old EV71- infected mice. The survival rates (A), body weight (B) and clinical scores (C) of mice were recorded. (D)In separate experiments, the hindlimb muscles (n = 6) of infected mice were collected on day 3 after infection to determine the IL-22 level. The relative change of IL-22 was compared with that of pre-infection mice. Data are mean SE values. *$P < 0.05$, by log-rank test (A), Wilcoxon test (C), and Mann-Whitney U test (D).

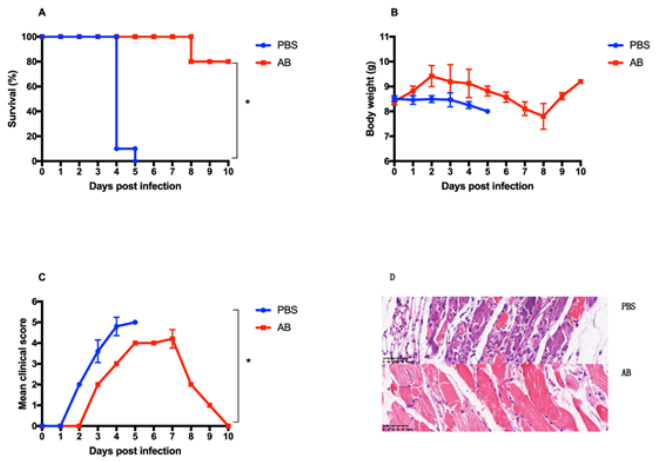

Figure 2. Reduction of the mortality and subsequent paralysis sequelae of EV71-infected mice by IL-22 neutralizing antibody, via reduction of inflammation and necrosis in tissues. Infected mice were treated with PBS (n = 10) or IL-22 neutralizing antibody (AB) (n = 10). The survival rates (A), body weight (B) and clinical scores (C) of mice were recorded. D, In separate experiments, the hind limb muscles of infected mice treated with PBS (n = 6) or IL-22 neutralizing antibody (n = 6) were collected on day 3 after infection to determine inflammation and necrosis. Data are mean SE values. **P* < 0.05, by log-rank test (A), Wilcoxon test (C).

day 7 after infection, while none of the PBS-treated mice survived upon day 5. Additionally, after weight recovery, EV71-related clinical symptoms in the antibody-treated mice disappeared and their motor ability was largely restored. Furthermore, we observed unilateral or bilateral ocular inflammation in mice infected with GD 10-12. In this infection model, the antibody-treated mice also indicated full recovery from eye inflammation. In conclusion, anti-IL-22 neutralizing antibody treatment significantly reduced mortality and morbidity in EV71 and GD 10-12 infected mice.

Our experiment indicated that the most significant symptom of EV71 infection is muscle lesions detected in the mice hind limbs. To investigate the mechanism of anti-IL-22 antibody-related reduction in mortality and clinical symptoms, histopathological and viral titer comparisons were performed in the muscle tissues of the hind limbs of control and IL-22 antibody-treated mice. Control mice showed significant inflammation and necrosis of the hind limb muscles on the 3rd day after infection. Alternatively, IL-22 antibody-treated mice demonstrated much less muscle inflammation and muscle tissue necrosis (Figure 2D). No significant differences were found in virus titer in muscle tissue between the two groups (data not shown).

Genetic analysis of hindlimb muscles RNA transcription in mice infected by EV71

Differences in RNA transcription levels were compared and analyzed in hindlimb muscles of 7, 14, 18 and 21-day-old mice before and after the introduction of EV71 infection. Gene transcription levels that were changed more than 2 folds were defined as differentially expressed genes. There were 3353, 4204, 3350, and 2193 differentially expressed genes in mice at the age of 7, 14, 18, and 21 days, respectively. The total number of differentially expressed genes was 7252. These genes were analyzed using cross-and-union differential set analysis (figure 3A). The results show that 337 differentially expressed genes were present in all mice despite of difference in ages.

Further expression value clustering analysis was carried out for these 337 genes mentioned above. Notably, the expression patterns of these genes in 1, 7, and 14-day-old mice were similar. The expression patterns of these genes were also similar in 18 and 21-day-old mice (figure 3B). However, there were fundamental differences in the expression pattern between the two groups. This result is consistent with the observation in the previous mice infection experiment. In the previous model, most of the strains could cause death in 1, 7, and 14-day-old mice, while the virus was rarely lethal in 18 and 21-day-old mice.

The differentially expressed 337 genes were analyzed using functional GO TERM clustering analysis (figure 3C). Our data shows that the majority of these gene functions were associated with regulations of basic life-supporting processes, virus-related immunity, and control of symbiotic microorganisms. Our study aimed to find the genetic changes that are most closely associated with the development and resolution of EV71 infection *in vivo*. Genes involved in support of basic life processes were considered as background. Stripping out the impact of these background genes, we found that some of the genes involved in viral immunity responses were the most actively differentiated (Figure 3C).

To determine the expression of these viral immunity-related genes, we sequenced them and analyzed the levels of their expression (Figure 3D). The top 3 highly expressed

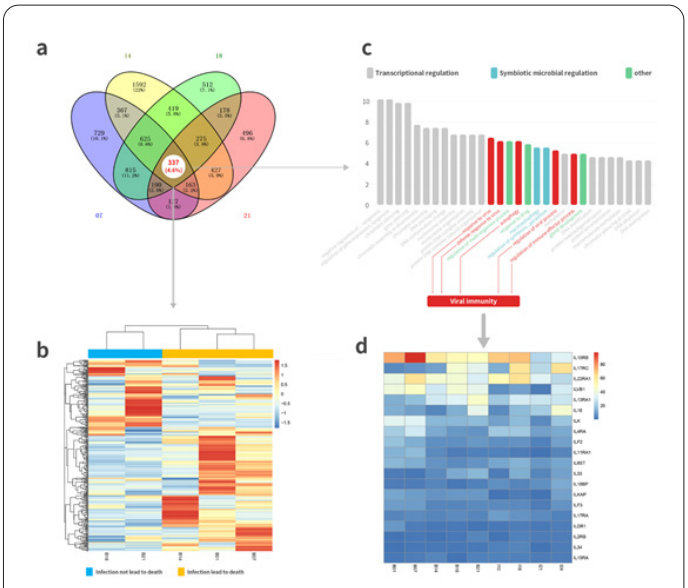

Figure 3. Genetic analysis of hindlimb muscles RNA in mice infected by EV71. (A) Venn diagram of the gene differences in the four different groups. Blue, yellow, green and red colors represent the number of differentially expressed genes in the 7-day-old mouse group, the 14-day-old mouse group, the 18-day-old mouse group and the 21-day-old mouse group, respectively. (B) Gene expression clustering diagram of the 337 differentially expressed genes in mice. The vertical axis is genes. The horizontal axis is mouse age by day B01, B07, B14, B18 and B21 represent mice samples of day 1, 7, 14, 18 and 21 respectively. Different colors represent different multiples, scaled by column. (C) Gene Ontology Enrichment of the 337 genes. The vertical axis is the proportion of enrichment genes (the total number of enriched genes/Gene ontologies). (D) Expression calorimetric map of cytokine family genes. The top 20 are displayed according to the maximum number of expressions.

genes were IL10 receptor B (IL10RB), IL-17 receptor C(IL-17RC), and IL-22 receptor A1 (IL-22RA1). These results suggest that these receptor-related cytokines may play an important role in the progression of EV71 infection. Following this, we tested the level of these cytokines (IL-10, IL-17, IL-22) expression in mice in several age groups. We found that IL-22 was differentially expressed in mice at different ages infected with EV71.

Discussion

The current study is the first to demonstrate the effectiveness of anti-IL-22 neutralizing antibodies in reducing inflammatory lesions in mouse tissue, along with morbidity and mortality of infected mice during acute EV71 infection. Considering that there is no effective treatment for fatal EV71 infection in humans, our data indicate a very promising approach and warrants future clinical testing.

We found that the application of anti-IL-22 neutralizing antibodies was effective against EV71 infection even 24 hours after the introduction of the viral components. Compared to our study, previous studies have shown that drug therapy must be initiated within 2h post-infection to achieve protective antiviral effects in mice (9). Continuous application of antiviral drugs for an extended period was found to be necessary to provide a protective effect during EV71 infection. Alternatively, we used an anti-IL-22 neutralizing antibody in mice for 5 days and observed a significant protective effect. Our data shows extended life expectancy in mice treated with anti-IL-22 after infection. To compare the effectiveness of this treatment with other antibodies, we used other cytokine-neutralizing antibodies in EV71-infected mice. The anti-IL-23 and anti-IL-1 neutralizing antibodies had a less pronounced protective effect in EV71-infected mice. The mortality rate of the infected mice was not effectively reduced.

During our study, anti-IL-22 neutralizing antibodies were found to help an infected host survive the acute inflammatory phase. IL-22 antibodies reduced the inflammatory response and tissue damage associated with the EV71-induced inflammation. In contrast to other tested antiviral drugs, IL-22 neutralizing antibodies targeted the pro-inflammatory response to EV71 infection, rather than viral replication or transmission. Therefore, the anti-IL-22 neutralizing antibody treatment may not be specific to virus strains or species. Suggestively, the antibodies might represent universal characteristics. However further testing is required to confirm the observed anti-inflammatory responses in models with other viruses causing HFMD. A larger panel of anti-viral testing is required to confirm our findings. Our findings are supported by previously found data. For instance, circulating Th22-producing cells have been identified in patients with psoriasis and were shown to contribute to cutaneous inflammation (15-22). An increased frequency of cTh22-producing cells in rheumatoid arthritis patients was positively correlated with rheumatoid factor and disease severity (11). These reports demonstrate that IL-22 may play important pro-inflammatory roles in various virus-linked diseases.

Importantly, our data has demonstrated that blocking or weakening the role of IL-22 in the process of EV71 infection can effectively inhibit the tissue damage caused by EV71 infection. In conclusion, IL-22 plays a very important role in promoting inflammation and aggravating injury in the disease process of EV71 infection. Therefore, our findings not only further elucidate EV71 pathogenesis, but also define a promising diagnostic indicator for severe cases of EV71 infection. However, the mechanism of IL-22 action and its relationship with HFMD severity warrants further study.

Conflict of interests

The authors declared no conflict of interest.

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