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# Pathologic and immunologic characteristics of coxsackievirus A16 infection in rhesus macaques

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ABSTRACT

Coxsackievirus A16 (CV-A16) causes human hand, foot and mouth disease, but its pathogenesis is unclear. In rhesus macaques, CV-A16 infection causes characteristic vesicles in the oral mucosa and limbs as well as viremia and positive viral loads in the tissues, suggesting that these animals reflect the pathologic process of the infection. An immunologic analysis indicated a defective immune response, which included undetectable neutralizing antibodies and IFN-γ-specific memory T-cells in macaques infected with CV-A16. Furthermore, existing neutralizing antibodies in macaques immunized with the inactivated vaccine were surprisingly unable to protect against a viral challenge despite the presence of a positive T-cell memory response against viral antigens. The virus was capable of infecting pre-conventional dendritic cells and replicating within them, which may correlate with the immunological characteristics observed in the animals.

## 1. Introduction

Recent pathogenic studies have shown that enterovirus 71 (EV-A71) and coxsackievirus A16 (CV-A16) are the two major causative agents of 90% of hand, foot and mouth disease (HFMD) cases (Li et al., 2005; Rabenau et al., 2010; Xing et al., 2014). Due to public health concerns, the control and prevention of HFMD epidemics is an emerging need that has substantially promoted the development of vaccines against both viruses (Kung et al., 2014; Li et al., 2014; Mao et al., 2014; Zhu et al., 2014). Pertinent pathogenic and immunological studies have shown promising results toward the development of a vaccine to prevent the emergence of more severe cases resulting from EV-A71 infections rather than other enteroviruses (Ang et al., 2009; Kim et al., 2014; Xing et al., 2014). The clinical and pathological characteristics of patients with CV-A16 infection and subsequent HFMD include mild manifestations characterized by vesicles in the oral mucosa and on the hands and feet (Ang et al., 2009; Jia et al., 2011; Mao et al., 2014). In the vast majority of cases, these clinical manifestations are accompanied by fever and flu-like symptoms (Xu et al., 2012; Zhu et al., 2007). Nevertheless, death from CV-A16 infection has been reported on rare occasions (Wright et al., 1963). Although the data on CV-A16 pathogenesis have mainly been derived from experiments using mice (Cai et al., 2013; Huang et al., 2015; Mao et al., 2012; Yang et al., 2014), several studies of infected children have indicated an imbalance among T-cell subsets and an abnormal up-regulation of cytokines following CV-A16 infection (Luo et al., 2015), suggesting that the interaction of the virus and host may induce a unique immune response. Interestingly, some studies have suggested that repeated CV-A16 infections may occur (Xie et al., 2013). These specific clinical characteristics and the unknown pathogenesis of this infectious disease have slowed the development of CV-A16 vaccines. Therefore, detailed analyses of the pathogenesis and immune responses induced by CV-A16 would provide the necessary data to develop a vaccine against this virus. Mouse studies have suggested that cerebral and intraperitoneal injections result in viral infection-mediated death accompanied by tissue lesions, including severe alveolar shrinkage, pulmonary fibrosis, edema and severe necrosis of the skeletal muscle. Moreover, the viral load slowly increases in various tissues during infection in association with an immune response characterized by neutralizing antibodies (Cai et al., 2013; Huang et al., 2015; Mao et al., 2012; Yang et al., 2014). However, in-depth studies of the pathogenesis of this infection in mice have not provided

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supporting data to advance the development of clinical treatment and a preventive vaccine. In the work presented here, the infectious process of CV-A16 was verified using rhesus macaques aged 6–8 months. The experimental results provide the essential pathological characteristics of the infection, including the presence of vesicles in the oral mucosa and viremia 4–7 days post-infection (p.i.); these are typical clinical symptoms of HFMD, even after the 2nd or 3rd repeat infection in the same individual. However, other than these clinical manifestations, no severe pathologic changes were observed in other organs or tissues during the infection. Further observation suggested that the virus is incapable of inducing a neutralizing antibody response, even after repeat infections. Therefore, no distinct immune protection against viral challenge was observed in infected individuals. *In vivo* and *in vitro* studies have suggested that the pathogenesis of this viral infection might involve a defective immune response; specifically, CV-A16 may prefer to infect a subset of conventional dendritic cells (cDC), which may be related to the observed immune dysfunction. This abnormal immunity induced during CV-A16 infection in macaques suggests that further investigations of the immunologic mechanism of CV-A16 infection are necessary to develop CV-A16 vaccines, including inactivated, attenuated and subunit vaccines.

## 2. Results

### 2.1. Pathologic characteristics of CV-A16-infected rhesus macaques

CV-A16 infection was previously studied in a mouse model. As an enterovirus, CV-A16 was able to induce pathologic death and neurogenic symptoms in the mouse (Mao et al., 2012), but vesicles, which are a classical feature of human disease, were not observed. In this study, twelve macaques infected with CV-A16 via nasal insufflation all presented the characteristic oral mucosa and limb vesicles associated with increases in body temperature (Fig. 1A), and peak viremia was observed in the peripheral blood samples of all animals within 3–9 days of viral challenge (Fig. 1B). The same viral peaks were found in the throat swabs and stool samples collected from these animals (Fig. 1B). Further histopathologic observations of the animals sacrificed by anesthesia 7 days after viral challenge failed to identify histopathologic events in most of the organs and tissues, although slight inflammatory cell aggregation in some lymph nodes of the respiratory tracts (Fig. 1C and D) and slight pathologic injury associated with this inflammatory infiltrate in the epidermal layer of the vesicle lesion site (Fig. 1E) were observed. However, viral proliferation was identified in the vast majority of organs, especially in some lymph nodes, the thyroid gland, chest muscles, the trachea and the esophagus (Fig. 1F). Additionally, these macaques exhibited increased ratios of monocytes and natural killer cells in the peripheral blood (Fig. 1G and H). These results may indicate that CV-A16 can infect macaques aged 6–8 months and cause clinical and pathologic manifestations that are characteristic of human HFMD cases, including vesicles and viremia.

### 2.2. Tracking CV-A16 in vivo during infection in macaques

Based on the clinical observations of CV-A16-infected macaques, we further tracked the virus *in vivo* in the macaques to clarify the pathologic process of the viral infection. Based on the result of the viral load assay of the trachea described in Fig. 1F, the first round of viral replication is believed to occur in the trachea. Further immunofluorescence analyses using confocal microscopy suggested that viral proliferation in the epithelial cells and pre-DCs of the trachea after infection, as indicated by the co-localization CV-A16 with green signal, cytokeratin 14 (epithelial cells) and CD11c (pre-cDCs) with red signals in the tracheas of sacrificed macaques 3 days p.i. (Fig. 2A and B). This pattern was consistent with the viral proliferation-mediated activation of the innate immune response and DC recruitment in the epithelial tissue of the trachea (Banchereau et al., 2000; Guidotti and Chisari, 2001). Additional virus tracking in the lymph nodes of the respiratory tracts of these sacrificed macaques 3 and 7 days p.i. indicated that the ratio of the virally infected $CD11c^+$ population in the DCs obtained on day 3 was higher than that in the DCs obtained on day 7 (Fig. 2C). Conversely, the ratio of the infected $CD83^+$ and $CD86^+$ populations within the DCs obtained on day 7 was higher than those within the DCs obtained on day 3 (Fig. 2C). A flow cytometric analysis of the $CD11c^+$ cells and $CD83^+$ and $CD86^+$ cells positive for virus isolated from the spleens of sacrificed macaques on days 3 and 7 also suggested a similar result (Fig. 2C). qRT-PCR using specific primers for the CV-A16 VP1 sequence confirmed the above observation (Fig. 2D) and also indicated that the $CD4^+$, $CD8^+$ and $CD3^+$ populations isolated from the same infected macaques were negative for viral replication. To confirm CV-A16 infection in DCs, cultured $CD11c^+$, $CD83^+$ and $CD86^+$ cells isolated from macaques were used to measure viral replication using a dynamic growth curve. The results indicated that CV-A16 was capable of proliferating in these cells, as evidenced by a typical dynamic growth curve and detection of the negative-strand RNA of the viral genome during infection (Fig. 2E and F). These data suggest that CV-A16 can enter and replicate within pre-conventional dendritic cells (pre-cDCs) in the epithelial tissue of the respiratory tract after infecting epithelial cells in order to stimulate DC maturation while also replicating in other cells during infection. To confirm this phenomenon observed in macaques, human $CD11c^+$ pre-DCs isolated from healthy individuals were cultured in vitro and infected with CV-A16. The results suggested that similar viral proliferation occurred in these cells, with a dynamic growth curve and the presence of negative-strand viral RNA (Fig. 2G and H).

### 2.3. CV-A16 infection fails to induce an effective immune response in macaques

Based on the clinical observations of macaques infected with CV-A16 in this study, subsequent investigations focused on the specific immune response after viral infection. To accomplish this, specific neutralizing antibodies against CV-A16 and the memory T-cell response against viral VP1 antigens were detected using micro-neutralization assay and IFN-γ-specific ELISPOT assay. Within at least 2 months of virus infection, neutralizing antibodies did not develop in the macaques. The highest neutralizing antibody titer was 1:16, whereas the majority of macaques possessed titers of 1:4 (Fig. 3A). The specific memory T-cell responses observed in the IFN-γ-specific ELISPOT assay were negative as CV-A16 VP1, VP2 and VP3 antigenic peptides were used to stimulate to the PBMCs from infected macaques within the same period (Fig. 3B). Additionally, the detection of pro-inflammatory cytokines in the peripheral blood samples of all animals revealed elevated IL-6 and TNF-α levels on day 7 (Fig. 3C and D). These data suggest a weak specific immune response in macaques infected with CV-A16 and prompted us to investigate a similar phenomenon in human patients. Forty-seven serum samples collected from children aged 6–72 months were used to detect neutralizing antibodies against CV-A16. These children were subjects of the placebo and vaccine groups in a phase III clinical trial of an EV-A71 inactivated vaccine (Trial registration number: Clinicaltrials.gov, NCT01569581) and were identified as being infected with CV-A16 because they exhibited typical features of HFMD, especially oral vesicular lesion and etiologic identification by qRT-PCR (Li et al., 2014). The results showed a neutralizing antibody titer of 1:4 in most children; the highest neutralizing antibody titer was 1:64, observed in a 36-month-old child. The GMT of all children was 4.18 (Fig. 3E). These data imply that CV-A16 infection may induce a weaker immune response in both macaques and humans.

### 2.4. CV-A16 can repeatedly infect macaques

The data obtained from the experiments described above suggested