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Coxsackievirus-B4E2 can infect monocytes and macrophages *in vitro* and *in vivo*

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ABSTRACT

Viral RNA (vRNA) is found in mice inoculated with coxsackievirus-B4E2 (CV-B4E2). The CV-B4E2 infection of murine spleen cells *in vitro* is enhanced with CV-B4E2-infected mouse serum. It has been investigated whether monocyte/macrophages were targets of CV-B4E2 in mice. vRNA has been detected in spleen and bone marrow of infected animals. The levels of vRNA were higher in CD14+ cells than in CD14- spleen cells and in F4/80- cells than in F4/80+ spleen cells. Meanwhile, CD14+ cells and F4/80- cells were more permissive to CV-B4E2 *in vitro* and the infection was enhanced when the virus was mixed with immune serum. While CV-B4E2 infected BMDM cultures (98% F4/80+); however, the immune serum did not enhance the infection. In conclusion, CV-B4E2 infects monocytes (CD14+, F4/80-) and macrophages (CD14+, F4/80+) *in vivo* and immune serum can enhance the *in vitro* infection of these cells arising out of the spleen.

1. Introduction

The *Enterovirus* genus of the *Picornaviridae* family consists of 12 species, Enterovirus A-H, J and Rhinovirus A-C. Coxsackieviruses B1-B6 (CVB) belongs to the Enterovirus B species; they are small, single stranded RNA viruses. The plus sense RNA is found inside a non-enveloped capsid composed of 60 protomers, each protomer is constituted of one single copy of the viral proteins VP1, VP2, VP3 and VP4 (Baggen et al., 2018). CVBs are common pathogens responsible for asymptomatic infections and for acute human diseases such as pancreatitis, myocarditis and meningitis. Furthermore, the role of CVB in chronic diseases such as type 1 diabetes (T1D) is suspected (Hober and Sauter, 2010; Hyöty, 2016). Epidemiological investigations strongly support the hypothesis of a relationship between enteroviruses and T1D (Hober and Sauter, 2010; Hober and Alidjinou, 2013; Bergamin and Dib, 2015). Markers of enteroviral infection have been found more frequently in patients with T1D than in controls (Richardson et al., 2009; Stene et al., 2010; Yeung et al., 2011; Oikarinen et al., 2011; Krogvold et al., 2015).

Macrophages are innate immune cells, involved in the defense against pathogens. During viral infection, macrophages release cytokines and chemokines that not only stimulate and recruit cells of the innate immune system, but also activate the adaptive immune system (Arango Duque and Descoteaux, 2014). Monocytes and macrophages derive from bone marrow (BM) myeloid progenitor cells. These myeloid progenitor cells give rise to monocytes that exit the BM and enter the circulation until they penetrate various tissues and differentiate into macrophages (Shi and Pamer, 2011). *In vitro* bone marrow cells can differentiate into macrophages when macrophage colony stimulating factor (M-CSF) is added to cultures (Weischenfeldt and Porse, 2008).

It was demonstrated that enteroviruses replicate in macrophages *in vitro* (Wahid et al., 2005) and enteroviral RNA was found in peripheral blood monocytes of patients with T1D (Alidjinou et al., 2015a). Human monocyte-derived macrophages can be infected by CV-B4 *in vitro* (Alidjinou et al., 2015b). Human monocytes are poorly infected with CV-B4 *in vitro* however the infection can be enhanced when the virus is incubated with non-neutralizing anti-CV-B4 IgG (Hober et al., 2001; Goffard et al., 2013). The antibody-dependent enhancement (ADE) of the infection of monocytes or macrophages with CV-B4 may play a role in the dissemination of the virus in the host, and consequently in the virus-induced pathogenesis of diseases induced by these viruses (Sauter and Hober, 2009). The role of monocytes and macrophages in the infection by CV-B4, *in vivo*, remains to be determined. First it is needed to address the issue of the infection of these cells in vivo. To this aim, an

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animal model is needed.

CV-B4E2 has been isolated from the pancreas of a deceased 10-year-old boy with diabetic ketoacidosis. Inoculation of CV-B4E2 in SJL inbred mice produced hyperglycemia, insulitis and beta cell death (Yoon et al., 1979). Outbred CD1 mice inoculated with CV-B4E2 did not become diabetic, but they were infected and harbored viral RNA in the spleen (Jaïdane et al., 2006; Precechtelova et al., 2015; Elmastour et al., 2016). Mouse spleen cell cultures are poorly infected with CV-B4E2 *in vitro* (Jaïdane et al., 2008). Nevertheless, as reported recently by our team, when the virus was incubated with diluted immune sera from CV-B4E2-infected CD1 mice, the infection of spleen cells *in vitro* was enhanced due to anti-CV-B4E2 IgG contained in serum, but the target cells of CV-B4E2 were not identified (Elmastour et al., 2016). The purpose of the present study was to determine whether monocyte/macrophages were targets of CV-B4E2 *in vitro* and *in vivo* in CD1 mice.

2. Material and methods

2.1. Virus

The diabetogenic CV-B4E2 strain was kindly provided by Ji-Won Yoon (Julia McFarlane Diabetes Research Center, Calgary, Alberta, Canada). This strain was isolated from the pancreas of a child with recently diagnosed type 1 diabetes and was diabetogenic in mice. The virus was propagated in HEp-2 cells, and after three freeze-thaw cycles the suspension was collected and clarified at 2000 g for 10 min at 4 °C. Aliquots of virus preparations were stored at − 80 °C.

The encephalomyocarditis virus (EMCV) EMC strain (ATCC) was propagated in L-929 cells. Supernatants were collected 3 days after inoculation, clarified by centrifugation at 2000 g for 10 min, divided into aliquots, and stored at − 80 °C.

2.2. Cell line

HEp-2 cells (BioWhittaker) were grown in minimum essential medium (MEM; Gibco BRL) supplemented with 10% of fetal calf serum (FCS, Sigma), 1% of L-glutamine (Gibco BRL), 1% of non-essential amino acids and 1% of penicillin and streptomycin (BioWhittaker). HEp-2 cells were used for the production and titration of the CV-B4E2 virus.

L-929 cells (kindly provided by T Jouault, Lille, France) were grown in Dulbecco's modified eagle's medium (DMEM; Gibco BRL) supplemented with 10% FCS, 1% L-glutamine (Gibco BRL), 50 µg/ml streptomycin, and 50 IU/ml penicillin (BioWhittaker).

2.3. Mice

Three-week-old CD1 male mice were obtained from Envigo RMS Sarl (Harlan Laboratories) and were used for *in vivo* infection studies, *in vitro* generation of BMDMs, isolation and *in vitro* culture of splenic cells.

All experiments were done in accordance with French and European guidelines for animal care, and were approved by the Ethical committee for animal experimentation of Nord-Pas-de-Calais (France).

2.4. In vitro generation of BMDMs

BMDMs were prepared from 3-week-old CD1 male mice femurs and tibias as previously described (Weischenfeldt and Porse, 2008). Briefly, bone ends were cut off and marrow was flushed with DMEM supplemented with 10% FCS, 1% non-essential amino acids, 50 µg/ml streptomycin, and 50 IU/ml penicillin and 1% L-glutamine (Gibco BRL). The cell suspension was then filtered through a 70 µm cell strainer (Falcon), and 5.10^7 cells/10 ml were seeded onto a 10-cm bacterial dish in a medium containing 10 ng/ml of M-CSF (Cell guidance systems). The medium was changed every 3–4 days. On day 10, the bone marrow cells were differentiated into macrophages. Thereafter, cells were scraped and seeded in a 6-well cell culture plate, at 5.10^6 cells/well.

2.5. Isolation of total splenic cells, and separation of CD14 and F4/80 subsets

Three-week-old CD1 mice were killed by cervical dislocation, and their spleens aseptically removed and used to prepare primary cultures of total spleen cells cultures as described previously (Zamzami et al., 1996). Cells were prepared on ice, depleted of erythrocytes by hypotonic shock, then suspended in RPMI-1640 (Eurobio) supplemented with 10% FCS, 1% L-glutamine, 50 µg/ml streptomycin, 50 IU/ml penicillin and 10^{-5} M β-mercaptoethanol (Sigma). Then, cells were seeded into 96-well culture plates at 5.10^5 cells per well and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

The CD14 and F4/80 population were positively selected using EasySep™ Mouse Biotin Positive Selection Kit (Stemcell technologies) according to the manufacturer instructions. Briefly, a spleen cell suspension of 2.10^7 cells in 500 µl medium was prepared in a 5 ml polystyrene tube. 10 µl/ml of the FcR blocking antibody was added to the suspension then the primary biotinylated antibody anti-CD14 (eBioscience) or anti-F4/80 (Biolegend) were added at a final concentration of 1.5 µg/ml and incubated for 15 min at room temperature. Next, 100 µl/ml of the EasySep® Biotin selection cocktail were added and incubated for another 15 min at room temperature. Finally, 50 µl/ml of premixed EasySep® Magnetic Nanoparticles were added and incubated at room temperature for 10 min. The cell suspension was brought to a total volume of 2.5 ml by adding the RPMI medium and the tube was placed in the magnet for 5 min. The supernatant fraction containing the non-labeled cells was poured off in another tube. The tube was removed from the magnet and 2.5 ml of RPMI were added and the cells were gently mixed by pipetting up and down and then the tube was placed back in the magnet to repeat the separation for 4 times. The tube was then removed from the magnet and the labeled cells re-suspended in RPMI medium.

2.6. Flow cytometry and fluorescence-activated cell sorting

CV-B4E2-infected mice and control mice inoculated with medium were sacrificed and their spleen harvested and processed as mentioned above. The Fc receptors on the cells were then blocked with an anti-CD16/32 antibody (Clone 93, Biolegend) and then stained for 30 min at 4 °C with antibodies mix. After a wash, cells were fixed in 2% paraformaldehyde for 10 min at room temperature. Cells were washed two times and resuspended in 500 µl of PBS solution. The following antibodies were used in flow cytometry: PE-labeled anti-F4/80, FITC-labeled anti-CD14 and APC-labeled CD11b (All from Biolegend). Flow cytometry was performed using a LSR Fortessa X20 (BD Biosciences). Cells were sorted with a BD FACS Aria III SORP (BD Biosciences) and data were analyzed with BD FACSDIVA software.

2.7. Mice inoculation

Three-week-old CD1 male mice were intraperitoneally inoculated with CV-B4E2 at 2.10^4 $TCID_{50}$ units in 200 µl of culture medium or with 200 µl of culture medium. Seven days post-infection, the mice were sacrificed by cervical dislocation then spleen, bone marrow and blood were collected.

2.8. Inoculation of cell cultures

Mice were inoculated with CV-B4E2 (2.10^6 $TCID_{50}$/ml) then on day 21 post inoculation the animals were sacrificed, blood was collected and centrifuged to obtain serum. The anti-CV-B4E2 neutralizing titer of serum samples ranged from 2048 to 4096. 2 µl of mouse serum of CV-B4E2 infected mice were diluted ($1/10^3$, $1/10^4$ and $1/10^5$) and 2 µl of serum from non-infected mice were diluted ($1/10^4$) in duplicate in