# Dendritic cells during mousepox: The role of delayed apoptosis in the pathogenesis of infection

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## ARTICLE INFO

## ABSTRACT

Dendritic cells (DCs) are effector cells linking the innate immune system with the adaptive immune response. Many viruses eliminate DCs to prevent host response, induce immunosuppression and to maintain chronic infection. In this study, we examined apoptotic response of dendritic cells during *in vitro* and *in vivo* infection with ectromelia virus (ECTV), the causative agent of mousepox. ECTV-infected bone marrow dendritic cells (BMDCs) from BALB/c mice underwent apoptosis through mitochondrial pathway at 48 h post infection, up-regulated FasL and decreased expression of anti-apoptotic Bcl-2 and pro-apoptotic Fas. Similar pattern of Bcl-2, Fas and FasL expression was observed for DCs early during *in vivo* infection of BALB/c mice. Both BMDCs and DCs from BALB/c mice showed no maturation upon ECTV infection. We conclude that ECTV-infected DCs from BALB/c mouse strain help the virus to spread and to maintain infection.

## 1. Introduction

Dendritic cells (DCs) are effector cells of the innate immune system employed early during viral infection to control the pathogen and limit its spread from the site of infection. These professional antigen presenting cells (APCs) are widely dispersed throughout the body and located in the main portals of the entry of microbes, such as the skin and mucosal epithelia. After the initial capture and processing of potential antigens, DCs undergo a maturation program that results in the upregulation of costimulatory molecules and then activate specific T and B lymphocyte effector mechanisms (adaptive immunity) [1,2].

Ectromelia virus (ECTV; family Poxviridae, genus Orthopoxvirus) is a large DNA virus and the causative agent of mousepox that is closely related to vaccinia virus (VACV) and variola virus (VARV) causing smallpox [3,4]. Following respiratory infection or in the footpad, ECTV infects the draining lymph nodes and spreads through the lympho-hematogenous route to seed visceral organs, mainly liver and spleen [3,4]. Survival following ECTV challenge requires recruitment of natural killer (NK) cells [5], the induction of CD8+and CD4$^+$ T cells [6], and production of antibodies by virus-specific B cells [7]. Dendritic cells become infected with ECTV in the draining lymph nodes and present viral antigens to naïve CD8$^+$ T cells [4]. Depletion of CD11c + cells confers susceptibility to lethal ECTV challenge, demonstrating that CD11c + cells are required for resistance [8]. However, Kaminsky et al. demonstrated that plasmacytoid dendritic cells (pDC) are also necessary for survival of ECTV challenge through production of IFN-α [9].

Apoptosis is an active process triggered by a variety of extrinsic and intrinsic signals such as cytokines, growth factors, reactive oxygen species, UV-light, chemical and infectious agents. Two major signaling pathways have been discovered: receptor-ligand mediated pathways (such as Fas/FasL pathway) and mitochondrial-driven pathway. These pathways are regulated by abundant and still growing number of pro- and anti-apoptotic molecules [10].

Many viruses use the strategy to eliminate DCs in order to prevent the response of the host, induce a state of immunosuppression and to maintain chronic infection [11]. We have previously shown that ECTV-infected DCs in ECTV-resistant C57BL/6 mice down-regulate expression of MHC I, MHC II and CD80 and show significant induction of apoptosis [12]. These results are consistent with other authors showing a lack of DC maturation during VACV infection [13]. Also modified vaccinia virus Ankara induces apoptosis in DC draining from the skin and this occurs within the first few hours after infection thus preventing efficient antigen

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presentation [14].

However, not all viral infections promote DC apoptosis. Some studies indicate suppression of apoptosis in cell cultures infected with human respiratory syncytial virus (HRSV), human meta-pneumovirus (HMPV) and human parainfluenza virus type-3 (hPIV3), which was probably a result of the activation of Bcl-2 family proteins during the process of maturation [15].

Poxviruses are large DNA viruses which inhibit or promote apoptosis at many points of the intracellular signal transduction chain. The first orthopoxvirus gene product found to be associated with inhibition of apoptosis was the cytokine response modifier A gene (crmA) from cowpox virus (CPXV) [16]. CrmA is one of three serine proteinase inhibitors (serpins or SPI proteins) encoded by poxviruses and is referred as SPI-2. SPI-2 is highly conserved among orthopoxviruses, such as ECTV, VACV, and VARV [17]. Ectromelia virus encoded SPI-2 was showed to inhibit *in vitro* caspase 1 and caspase 8 but not granzyme B [18]. Many poxviruses also encode mitochondrial anti-apoptotic proteins, such as VACV-encoded F1L protein, myxoma virus (MYXV) encoded M11L or ECTV EVM025 protein [19–21]. These proteins localise to the mitochondria and inhibit cytochrome c release in response to a wide variety of apoptotic stimuli through inhibition of pro-apoptotic proteins [21–23].

Here, we found that ECTV-infected DCs from BALB/c mice undergo delayed apoptotic response both during *in vitro* and *in vivo* infection. Furthermore, upon ECTV infection, dendritic cells do not undergo maturation and up-regulate FasL receptor with decreased expression of Fas. Such phenotype of dendritic cells may help the virus to reach the local lymph nodes and shape the onset and type of the systemic infection.

## 2. Materials and methods

### 2.1. Virus

ECTV-MOS strain (ATCC, VR-1374) was grown and titrated in Vero cells and prepared by one cycle of freeze-thawing and subsequent removal of cellular debris by centrifugation. The aliquots of the stock ($10^6$ PFU/ml) were snap frozen and stored in liquid nitrogen.

### 2.2. Apoptosis and apoptotic protein detection

Apoptosis in the single cell suspensions was detected using Annexin V-Apoptosis detection kit I (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. Changes in the mitochondrial potential were detected with a cationic dye, 5,5',6,6'-tetrachloro1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) (Sigma-Aldrich) Briefly, the cells were incubated with culture medium (RPMI-1640 or α-MEM) containing 10% FBS and 5 µg/ml JC-1 at 37 °C for 15 min. Following incubation, cells were washed two times in PBS and analyzed in FACS Calibur using CellQuest programme (Beckton Dickinson Franklin Lakes, NJ, USA) for the percentage of cells with a decrease in the red to green fluorescence intensity ratio.

For detection of Fas and FasL, cells were washed in 1% FBS/PBS, then FITC-conjugated hamster anti-mouse Fas antibody (Jo-2) and PE-conjugated hamster anti mouse FasL antibody (MFL4) were used together with (BD Biosciences). Intra-cellular antigens were detected using Cytofix/Cytoperm fixation/permabilisation kit (BD Biosciences) and the following antibodies: PE-conjugated mono-clonal hamster anti-Bcl-2 antibody (BD Biosciences), polyclonal rabbit anti-Bax antibody (BD Biosciences) and PE-conjugated polyclonal rabbit anti-active caspase-3 antibody (C92-605) (BD Biosciences) antibody. Following incubation with primary

antibodies, appropriate anti-rabbit PE-conjugated antibodies were used, where necessary (BD Biosciences). For all stainings, appropriate isotype control antibodies were used. All stainings were analyzed at the FACS Calibur (BD Biosciences).

### 2.3. Bone-marrow derived dendritic cells

Primary cultures of bone marrow derived dendritic cells (BMDCs) were prepared by culturing bone marrow cells isolated from BALB/c mice strain in the D-MEM (Life Technologies) culture medium supplemented with 25 ng/ml GM-CSF and 15 ng/ml IL-4 (Life Technologies), 4500 mg/L glucose, antibiotics (penicillin and streptomycin), L-glutamine, 10% fetal bovine serum (Life Technologies) for 5 days. At day 5 primary cultures of dendritic cells were washed and subjected to infection with ECTV-MOS strain at MOI = 5 or with inactivated ECTV-MOS, incubated for up to 48 h and then harvested by scraping.

### 2.4. Mice and infection

Mice of both sexes, 5- to 7-week old, were used for all experiments. BALB/c mice were purchased from the Mossakowski Medical Research Centre (Warsaw, Poland). This study was performed in accordance with recommendations of the Polish Act of 21 January 2005 on animal experiments (OJ no 33, item 289) and Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes. The protocol was approved by the 3rd Local Committee on the Ethics of Animal Experiments in Warsaw, Poland. Mice were infected with ECTV-MOS diluted in PBS to the required dose ($5 \times 10^2$ PFU) via footpad route. Mice were observed every day for morbidity and mortality. At 5, 10 and 15 day post infection (d p.i.), infected and control mice were anesthetized and spleen and inguinal lymph nodes were isolated. Next, spleens and lymph nodes were passed through a 100 µm nylon mesh and washed in PBS/1%FBS. Each experimental group consisted of 5 animals, the experiments were repeated three times. Cell suspensions were prepared from pooled 5 spleens or lymph nodes per each mouse strain and experimental time point.

### 2.5. Cell characterization by flow cytometry

Cell suspensions were pretreated with the Fc receptors block - rat anti- CD16/32 antibody (2.4G2) (BD Biosciences) according to the manufacturer's protocol. For detection of activation markers, cells were washed in 1% FBS/PBS, then hamster anti-CD11c-APC (HL3) was applied together with one of the following antibodies: mouse anti- MHC Class I-FITC (34-1-2S), rat anti-I-A-PE (M5/114.15.2), hamster anti-mouse CD80-PE (16-10A1), rat anti-mouse CD86-FITC (GL-1) and hamster anti-mouse CD40-FITC (HM40-3) (e-Biosciences, Hatfield, United Kingdom). For all stainings, rat IgG2a, rat IgG2b and hamster IgG1 isotype antibodies conjugated with appropriate fluorochromes were used (e-Biosciences, Hatfield, United Kingdom). Following staining, cells were washed and analyzed in FACS Calibur for CD11c positively stained cells and co-expression of other markers.

### 2.6. Immunofluorescent staining

ECTV-MOS antigens were detected by rabbit polyclonal antibody raised against Vaccinia Virus VACV (Abnova, Taiwan), followed by a second-step with FITC-conjugated anti-rabbit polyclonal antibody (Sigma-Aldrich). Bcl-2, Bax, Fas and FasL were detected using the antibodies given above. After mounting, the slides in medium containing Hoechst 33342 (1 µg/ml), fluorescence