FEMS Pathogens and Disease, 73, 2015, ftv088

doi: 10.1093/femspd/ftv088
Advance Access Publication Date: 15 October 2015
Research Article

RESEARCH ARTICLE

The generation of CD8$^+$ T-cell population specific for vaccinia virus epitope involved in the antiviral protection against ectromelia virus challenge

Malgorzata Gierynska[1,*], Lidia Szulc-Dabrowska[1], Tomasz Dzieciatkowski[2], Anna Golke[1] and Ada Schollenberger[1]

[1]Department of Preclinical Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences – SGGW, Ciszewskiego 8, 02-786 Warsaw, Poland and [2]Chair and Department of Medical Microbiology, Medical University of Warsaw, Chalubinskiego 5, 02-004 Warsaw, Poland

*Corresponding author: Department of Preclinical Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences – SGGW, Ciszewskiego 8, 02-786 Warsaw, Poland. Tel: +48-22-5936062; E-mail: malgorzata_gierynska@sggw.pl

One sentence summary: Vaccinia virus derived TSYKFESV epitope-TLR9 agonist approach delivered systemically or mucosally, generated CD8$^+$ T cells that remained functional for at least 2 months, and cross-protected mice challenged with ectromelia virus.

Editor: Alfredo Garzino-Demo

ABSTRACT

Eradication of smallpox has led to cessation of vaccination programs. This has rendered the human population increasingly susceptible not only to variola virus infection but also to infections with other representatives of *Poxviridae* family that cause zoonotic variola-like diseases. Thus, new approaches for designing improved vaccine against smallpox are required. Discovering that orthopoxviruses, e.g. variola virus, vaccinia virus, ectromelia virus, share common immunodominant antigen, may result in the development of such a vaccine. In our study, the generation of antigen-specific CD8$^+$ T cells in mice during the acute and memory phase of the immune response was induced using the vaccinia virus immunodominant TSYKFESV epitope and CpG oligodeoxynucleotides as adjuvants. The role of the generated TSYKFESV-specific CD8$^+$ T cells was evaluated in mice during ectromelia virus infection using systemic and mucosal model. Moreover, the involvement of dendritic cells subsets in the adaptive immune response stimulation was assessed. Our results indicate that the TSYKFESV epitope/TLR9 agonist approach, delivered systemically or mucosally, generated strong CD8$^+$ T-cell response when measured 10 days after immunization. Furthermore, the TSYKFESV-specific cell population remained functionally active 2 months post-immunization, and gave cross-protection in virally challenged mice, even though the numbers of detectable antigen-specific T cells decreased.

Keywords: ectromelia virus infection; vaccinia virus TSYKFESV epitope; systemic model; mucosal model; cross-protection

INTRODUCTION

Over 200 years ago, Edward Jenner published his observations that infection with the animal poxvirus may protect humans against smallpox (Jenner 1798). That phenomenon, known as heterologous immunity, is explained by cross-reactivity between the two pathogens and this can happen between related and unrelated pathogens (Welsh 2002; Tscharke *et al.* 2005; Mathurin *et al.* 2009). Smallpox eradication was facilitated by the fact that variola virus (VARV) only affected humans and had no vector or carrier. However, with none now vaccinated against VARV, the

Received: 10 July 2015; **Accepted:** 5 October 2015

human population has become susceptible and as such a potential target for bioterrorism as well as infection by some animal poxviruses (Jacobs *et al.* 2009). Those include cowpox virus (CPXV), which is responsible for several zoonoses that occurred in Europe and Asia, monkeypox virus (MPXV), which circulates mainly in wild animals in Africa and caused the outbreak in 2003 in the mid-western United States due to the inadvertent importation of MPXV in a shipment of rodents from West Africa, and vaccinia virus (VACV), responsible for infection in dairy cows and dairy workers in Brazil (Henderson 1999; Enserink 2003, 2004; Vorou, Papavassiliou and Pierroutsakos 2008; Ninove *et al.* 2009; Quixabeira-Santos *et al.* 2011; Schatzmayr *et al.* 2011). Currently, there is a concern about continuing to use live VACV vaccine as this live virus generates mild to severe post-vaccination adverse effects. Moreover, animal and limited human data suggest that the third and fourth generation replication-defective VACV vaccines are less immunogenic than prior vaccine generations so the new approaches are needed for smallpox vaccination protocols (Rosenthal *et al.* 2001; Fulginiti *et al.* 2003; Halsell *et al.* 2003; Enserink 2004; Poland, Grabenstein and Neff 2005; Garcel *et al.* 2012;). Recent studies identified the human T-cell determinants of VACV but the alternative vaccination schema would not be developed using humans. However, the mouse poxvirus—ectromelia virus (ECTV)—does represent a valuable natural model since it is responsible for ectromelia in mice, a disease, closely resembling smallpox in humans (Fenner and Buller 1997; Esteban and Buller 2005). ECTV is highly infectious to all strains of laboratory mice, but induces lethal disease only in susceptible animals with particular genetic backgrounds (e.g. BALB/c (H-2^d), A/J (H-2^a), DBA/2(H-2^d)), and is readily cleared in other strains (e.g. C57BL/6 (H-2^b), AKR (H-2^k)), that are considered resistant (Fenner and Buller 1997; MacFadden 2005). Both VARV and ECTV encode viral IFN-γ binding protein (IFN-γbp), with the amino acid sequence similarity to the extracellular binding domain of IFN-γ receptor 1, and target host IFN-γ (Smith 2002; Sakala *et al.* 2007). Mechanisms involved in clearance of the virus usually involve T-cell-mediated immunity with engagement of IFN (α/β and γ), IL-12 p40 and IL-2 (Chaudhri *et al.* 2004). Several $CD8^+$ T-cell-restricted VACV epitopes have been described for C57BL/6 and BALB/c mice (Tscharke *et al.* 2005, 2006; Moutafasi *et al.* 2006, 2009). One of the dominant H-$2K^b$ restricted CD8 epitopes is derived from the viral IFN-γbp encoded by VACV B8R gene—$B8R_{20\text{-}27}$ (TSYKFESV). This protein is synthesized commonly by orthopoxviruses as a homolog of cellular receptors and can be detected within infected cells or as a secreted molecule. The ability of IFN-γbp to bind host IFN-γ influences the outcome of the immune response and allows orthopoxviruses to establish infection. Since it is also responsible for the virulence, this particular peptide combined with a proper adjuvant seems to be a good target for stimulating protective cell-mediated immunity (Nuara *et al.* 2006; Sakala *et al.* 2007; Alcami, Hill and Koszinowski 2010).

A novel group of adjuvants, synthetic oligodeoxynucleotides (ODNs) that contain unmethylated CpG motifs mimicking bacterial DNA, exert powerful adjuvant capacity and stimulate the immune response in favor of Th1 type and proinflammatory cytokine production (Krieg 2002; Bode *et al.* 2011). It has been proved that when used with proteins or peptides, the CpG ODNs can enhance resistance against infectious diseases by inducing immunity to individual epitopes recognized by $CD4^+$ or $CD8^+$ T cells (Gallichan *et al.* 2001; Gierynska *et al.* 2002; Mendez *et al.* 2003; Klinman 2004; Belyakov *et al.* 2006; Bode *et al.* 2011).

Stimulation of the adaptive immune response relays on the presence and ability of the antigen presenting cells (APCs), especially dendritic cells (DCs), to capture, process and present antigen to the naïve T cells in lymphoid tissue. DCs originate from the common DC progenitor, classified as conventional (or classical) DCs and plasmacytoid DCs (pDCs). The DCs subsets can be found in lymphoid tissues as well as at the mucosal sites where they are indispensable in the balance regulation between immunity and tolerance preventing the development of autoimmune diseases (Naik *et al.* 2007; Onai *et al.* 2007; Steinman 2007; Lewis 2012; Chung *et al.* 2013).

In the present report, we used the immunodominant TSYKFESV epitope derived from VACV along with the CpG ODNs adjuvant to determine if $CD8^+$ T cells cross-reaction to ECTV could be induced in mice using both systemic and mucosal models. Additionally, viral challenge studies were done to determine if the functional protective immunity developed and persisted. Our results show that a combination of the VACV derived TSYKFESV epitope and the bioactive CpG 1826 administered either systemically or mucosally, induced strong $CD8^+$ T-cell response when measured 10 days after immunization, and it persisted for at least 2 months. The differences observed in the level of induced immunity between both models during the acute phase of the immune response could result from transient silencing of immune response by pDCs in mucosal model. Immunized animals were protected against challenge with ECTV, even though the numbers of detectable antigen-specific $CD8^+$ T cells decreased in time. Our results are discussed in terms of additional procedures worth pursuing to improve the duration of immunity against ECTV in mice.

MATERIALS AND METHODS

Animals

Male C57BL/6 (H-2^b) 8-week-old mice were purchased from the animal facility at Maria Sklodowska Curie Memorial Cancer Center and Institute of Oncology in Warsaw, Poland. All experiments were done in compliance with the institutional Guidelines for Care and Use of Laboratory Animals. All experimental procedures on animals were approved by the 3rd Ethical Committee for Animal Experimentation at Warsaw University of Life Sciences—SGGW (permission no. 60/2009). Animal facilities at the Faculty of Veterinary Medicine (registration no. 14313537), are fully certified by the district veterinary inspector.

Reagents and culture media

Endotoxin-free CpG oligonucleotides: the bioactive CpG 1826 (5′-TCC AT **GACGTT** CCT **GACGTT**-3′ [immunostimulatory motifs are in boldface]), and the negative control CpG (5′-TCC ATG AGC TTC CTG AGC TT-3′) were purchased from InvivoGen. The peptides derived from VACV B8R (amino acids [aa] 20–27)—TSYKFESV and herpes simplex virus 1 glycoprotein B (HSVgB) (aa498-505)—SSIEFARL (used for specificity control), were synthesized and supplied by Lipopharm (Poland). Phorbol 12 myristate 13-acetate and ionomycin were used for the nonspecific $CD8^+$ T cells stimulation (PMA/Iono; Sigma). Ectromelia virus Moscow strain (ECTV-Mos) used in these studies was prepared as described previously (Gierynska *et al.* 2009). The following monoclonal antibodies (mAb): CD8a, gamma interferon (IFN-γ) and CD16/CD32, either conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin (APC) or purified (BD Pharmingen), were used for fluorescence activated cell sorter (FACS) staining. The Cytofix/Cytoperm kit (BD Pharmingen) was used for

intracellular detection of IFN-γ. Major histocompatibility complex (MHC) class I ($H2^b$) TSYKFESV pentamers (B8R-pentamer), conjugated with PE to quantify $CD8^+$ T cells TSYKFESV specificity, were provided by Proimmune Ltd. The subsets of pDCs and $CD11c^+$DCs were purified using magnetic separation, with anti-appropriate cellular antigen mAb conjugated with microbeads (Miltenyi Biotec). The presence of viral antigen was determined with the polyclonal rabbit ECTV-specific antibodies conjugated with FITC (ECTV-FITC pAb) and for the DNA visualization 4′,6-diamidino-2-phenylindole (DAPI) labeling was applied. Total RNA isolation kit (A&A Biotechnology) was used for RNA isolation from purified pDCs and $CD11c^+$DCs. Freshly isolated cells from mice spleen and draining lymph nodes (DLNs) and Vero cells were cultured as described previously (Gierynska *et al.* 2002).

Immunization protocol

In all experiments, if not said otherwise, C57BL/6 mice were immunized using either systemic route—injection in the hind footpads (fp) or mucosal route—intranasal application (in), with 100 μg of the TSYKFESV peptide combined with either 30 μg of the bioactive CpG 1826 or 30 μg of the negative control CpG. For the further experiments, cells were harvested from DLNs (popliteal and inguinal—the systemic immune response model or cervical—the mucosal immune response model, respectively) and from the spleen. For priming the cytotoxic TSYKFESV-specific $CD8^+$ T cells in the spleen and DLNs, mice were immunized twice: on day 0 and 10 either in the hind footpad or intranasally. Then 10 days (the acute phase of immune response) or 60 days (the memory phase of immune response) after the second immunization mice were sacrificed by cervical dislocation and DLNs and spleen were aseptically collected. For the virus challenge studies animals were intraperitoneally (ip) infected with 10^5PFU of ECTV-Mos/mouse.

To evaluate the role of DCs in stimulation of the adaptive immune response, C57BL/6 mice were immunized once with 100 μg of the TSYKFESV peptide combined with either 30 μg of the bioactive CpG 1826 or 30 μg of the negative control CpG using either systemic (fp) or mucosal (in) route of stimulation. Exactly 18 h post-induction all mice were sacrificed, and popliteal, inguinal or cervical DLNs were removed to obtain the pure populations of DCs.

Preparation of the cell suspension

The lymphoid cell suspensions from spleen and DLNs were obtained according to the procedures described previously (Gierynska *et al.* 2002). The popliteal, inguinal (systemic model) or cervical (mucosal model) LNs, the source of pDCs and $CD11c^+$DCs, were pressed through a metal sieve and then cells were washed once in RPMI 1640. For the enzymatic disaggregation, cells were suspended in Collagenase D solution (2mg/ml) for 30 min in 37° C. Subsequently they were washed twice with MACS buffer (0.5% bovine albumin serum and 2 mM EDTA in PBS), and magnetic separation was performed according to the manufacturer's protocols using anti-mPDC-1 microbeads (Miltenyi Biotec). Next, the rest of cells devoid of pDCs were incubated with anti-CD11c microbeads, and after incubation at 4°C the magnetic separation was again performed according to the manufacturer's protocols (Miltenyi Biotec). The purity of pDCs and $CD11c^+$DCs populations was confirmed using flow cytometry (data not shown).

The assessment of the $CD8^+$ T-cell population specificity and responsiveness to the antigen stimulation

To enumerate the IFN-γ producing cells, the intracellular cytokine (ICG) staining was performed using Cytofix/Cytoperm kit (BD Pharmingen) according to the procedures described previously (Gierynska *et al.* 2002). To assess antigen specificity of the generated $CD8^+$ T-cell population, a total of 10^6 cells isolated from spleen and DLNs were washed once in staining buffer and then resuspended in the residual solution (~50 μl). Exactly 10 μl of labeled B8R-pentamers were added and cells were incubated at the room temperature for 10 min, shielded from light. Subsequently, cells labeled with B8R-pentamers were washed twice with staining buffer and labeled with anti CD8 mAb. Double-color fluorescence was acquired and analyzed in a FACSCalibur flow cytometer and CellQuest software, respectively. The peptide-untreated cells and cells pulsed with the SSIEFARL peptide served as specificity controls. Cells stimulated with PMA/Iono (50 ng/ml and 500 ng/ml, respectively) served as the nonspecific positive control.

Challenge model

C57BL/6 mice were immunized twice, either systemically or mucosally, 10 days apart as described earlier. Then 10 days (the acute phase of immune response) or 60 days (the memory phase of immune response) after the second immunization animals were ip challenged with 10^5PFU of ECTV-Mos/mouse. Challenged mice were sacrificed by cervical dislocation on the second and fourth day post-infection, and tissues were collected for the viral load assessment (spleen) and $CD8^+$ T cells antigen specificity and the ability of IFN-γ production evaluation (spleen and DLNs). The presence of the ECTV antigen in the isolated organs was established by labeling with ECTV-FITC pAb and the nuclear DNA was demonstrated by counterstaining with DAPI. To evaluate the viral load in spleen, the virus reisolation was done on a 24 h Vero monolayer in the 24-well flat bottom plates. Briefly, spleens were collected and weighed, and then homogenized in DMEM supplemented with 1.5% (vol/vol) hi-FBS, 1.5 mM L-glutamine and antibiotic antimycotic to make a 10% (weight/volume [w/vol]) suspension, from which the 10-fold dilutions were prepared. Growth medium was aspirated from Vero cells and the appropriate dilutions of the spleen homogenates were added to a Vero monolayer in duplicates per dilution. Adsorption was performed for 45 min with a gentle agitation every 15 min and then the inocula were removed and after a brief rinse with DMEM without serum Vero cells were covered with a 2% low melting point agarose (LMP) solution in 2X DMEM without neutral red, supplemented with 2X regular additions. Once the LMP agarose solidified, plates were inverted and incubated in 37°C with 5% CO_2 for 5 days. Plaques were visualized by adding 2% LMP agarose solution in 2X DMEM supplemented with 2% of neutral red. Titer of the reisolated ECTV was expressed as the plaque forming units (PFU)/g of spleen tissue.

Detection of IL-10 and IL-10Rβ transcripts

To evaluate the role of DCs during the early phase of the immune response, C57BL/6 mice were immunized once as described previously. Approximately 18 h post-immunization mice were sacrificed by cervical dislocation and then popliteal, inguinal or cervical DLNs were collected to obtain the pure populations of pDCs and $CD11c^+$DCs using magnetic separation.

Table 1. Primers used in qPCR.

Target gene	Amplicon size	Forward primer	Reverse primer
GAPDH NM_008084.2*	205 bp	GCAAGGTCATCCCAGAG	AAGTCGCAGGAGACAAC
β-2-M NM_009735.3	150 bp	GTGGCATGAAGAAGGTGT	TGGAAATGGCAGAAGAAAGAT
IL-10Rβ NM_008349.5	186 bp	CAAACTAAGCATCATCAGCGA	AGGTGGCTCTCTGGTCATA
IL-10 NM_010548.2	153 bp	GTGAGAAGCTGAAGACCC	AGATGTCAAATTCATTCATGGC

*NCBI reference sequence.

Approximately 10^4 of freshly isolated cells, either pDCs or CD11c$^+$DCs, were immediately suspended in Trizol for the total RNA isolation. RNA obtained with Total RNA isolation kit (A&A Biotechnology) was analyzed for *IL-10* and *IL-10R*β mRNA expression using real-time PCR (qPCR). Complementary DNA was obtained according to the method described previously (Gierynska *et al.* 2009). Primers targeting the highly conservative region of analyzed genes (Table 1) were designed using the LightCycler Probe Design 2 software (Roche Diagnostics). The sequence specificity was confirmed *in sillico* by nucleotide-nucleotide search in the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST/). qPCR was performed using the LightCycler 2.0 instrument (Roche Diagnostics) and the final 20 μl volume contained: 2 μl of a template, 20 pM of the sense and antisense primers, 5 mM of $MgCl_2$ and the reaction mix of LightCycler® FastStart DNA Master SYBR GreenI (Roche Diagnostics). Amplifications were carried out subsequent to the thermostable hot-start DNA polymerase for 10 min at 95°C, followed by 45 cycles comprising denaturation (95°C for 15 s), primers annealing (60°C for 7 s), strand elongation (75°C for 6 s) and the last step of cooling to 40°C for 60 s. To establish the specificity of amplified products, the melting curve analysis was performed. Fluorescence levels were read at 530 nm wavelength specific for the SYBR GreenI dye. The gene expression was quantified by the $\Delta\Delta$Ct method. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-2 microglobulin (β-2-M) genes served as the internal controls used for standardization of obtained data.

Statistical analysis

All quantitative data were expressed as mean ± SD (standard deviation) from at least three independent experiments. Differences between groups of animals at indicated time points were calculated using the Student's *t*-test (in case of normal data distribution with equal variance) or Mann–Whitney U-test (STATISTICA 6.0 software, StatSoft, USA). As a limit for statistical significance $P \leq 0.05$ was used.

RESULTS

The immune response stimulation as a result of the systemic and mucosal immunization with the VACV-derived immunodominant peptide and the bioactive CpG ODN

In all experiments, if not said otherwise, C57BL/6 mice (H-2^b) were immunized twice, on day 0 and 10 with the immunodominant TSYKFESV peptide together with either the bioactive CpG 1826 or the negative control CpG using either systemic (fp) or mucosal (in) route. Then 10 days (the acute phase of immune response) or 60 days (the memory phase of immune response) post second immunization, DLNs and spleen were collected to measure the functional capacity of peptide-specific CD8$^+$ T cells. The following major points are to be noted.

First, generated CD8$^+$ T-cell responses, measured by the B8R-pentamer labeling and the specific peptide-induced intracellular IFN-γ production, were evident and significantly different from results obtained in control groups ($P \leq 0.05$), only in DLNs and spleen of mice immunized with the immunodominant TSYKFESV peptide and the bioactive CpG 1826 (Fig. 1, Fig. 3A and B). Moreover, the cytokine response measured by ICG indicated that both routes of immunization yielded functionally equivalent peptide-specific CD8$^+$ T-cell responses (Fig. 3A and B). No generation of CD8$^+$ T cells responsive to the *in vitro* stimulation with the TSYKFESV peptide was observed in mice immunized with only the bioactive CpG 1826 or peptide alone. Additionally, the generated TSYKFESV-specific CD8$^+$ T cells were not responsive upon *in vitro* stimulation with the SSIEFARL peptide (data not shown).

Second, the systemic route of immunization was more effective when assessed during the acute phase of the immune response. This route of stimulation generated more CD8$^+$/IFN-γ^+ T cells in the spleen during the acute phase (13.27 ± 1.55% what corresponds to a total cell number of 1.148 ± 0.13×10^6) than the mucosal route (8.93 ± 0.53% what corresponds to a total cell number of 0.49 ± 0.02×10^6). However, the level of responses was comparable irrespectively of the immunization route when measured in DLNs (systemic model—1.58 ± 0.5% [0.07 ± 0.02 × 10^6 of a total cell number]; mucosal model—1.71 ± 0.18% [0.12 ± 0.01 × 10^6 of a total cell number]) (Fig. 1, Fig. 3A and B).

Third, the induced immunity analyzed during memory phase of the immune response showed that the systemic and mucosal routes resulted in antigen-specific CD8$^+$/IFN-γ^+ T-cell response only in mice immunized with the TSYKFESV peptide and the bioactive CpG 1826 (Fig. 2, Fig. 3C and D). However, the frequency of the TSYKFESV-specific CD8$^+$ T cells in DLNs and spleen was lower in comparison to the frequency obtained during the acute phase of the immune response (Fig. 3B and D). There was a statistically significant decrease ($P \leq 0.05$) of the CD8$^+$/IFN-γ^+ T-cell population in both models, systemic and mucosal. The 5.3-fold decrease of CD8$^+$/IFN-γ^+ T cells frequency in mice spleen and the 3.3-fold decrease of CD8$^+$/IFN-γ^+ T cells percentage in DLNs were noticed in mice immunized systemically. However, in mice immunized mucosally the 3.8-fold decrease of CD8$^+$/IFN-γ^+ T cells percentage in spleen and 1.8-fold decrease of the TSYKFESV-specific CD8$^+$ T cells percentage in DLNs was observed, respectively. It was correlated with the decrease of CD8$^+$/B8R-pentamer$^+$ T cells (Fig. 2, Fig. 3C and D).

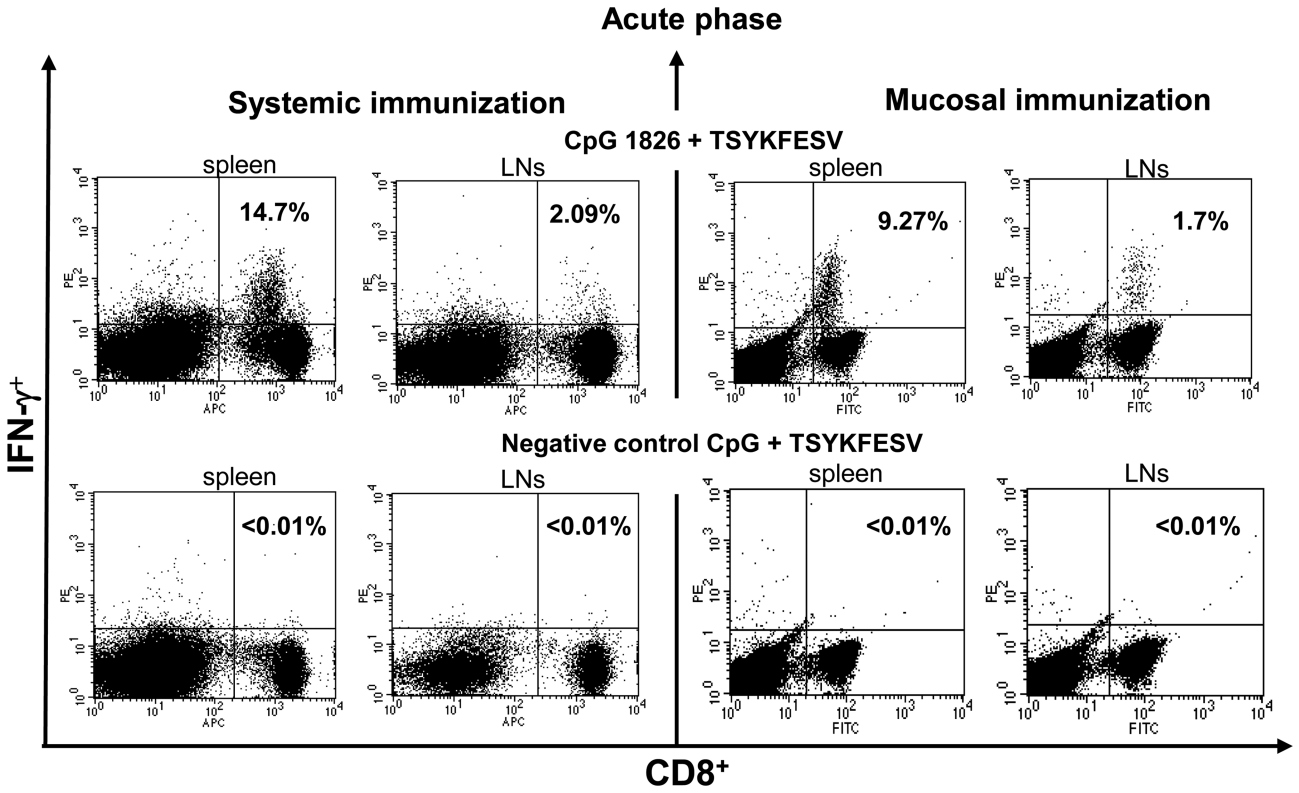

Figure 1. Generation of IFN-γ producing CD8$^+$ T cells in spleen and DLNs of C57BL/6 mice as a result of systemic or mucosal immunization with the TSYKFESV peptide and the bioactive CpG 1826—the acute phase of immune response. C57BL/6 mice were immunized twice, on day 0 and 10, either systemically (fp) or mucosally (in) with the immunodominant TSYKFESV peptide and either the bioactive CpG 1826 or the negative control CpG. Ten days post second immunization DLNs and spleen were collected. Obtained cells were stimulated with 1μg of the TSYKFESV peptide/10^6 cells and 50 U of rIL-2 in the presence of 1 μg of brefeldin A for 5 h at 37°C in the presence of 5% CO_2. Subsequently, the CD8$^+$ T-cell functionality was assessed by intracellular IFN-γ staining. Flow cytometry analysis was performed on cells isolated from the individual spleen and pooled DLNs in each experimental group. The representative graphs show the CD8$^+$/IFN-γ^+ T-cell populations (upper right quadrant) isolated from C57BL/6 mice during the acute phase of the immune response. The numbers within analyzed quadrants represent the percentage of positive cells for a given marker within the gate for lymphocyte population as defined by FSC and SSC parameters.

Our data indicate that combination of the TSYKFESV epitope derived from VACV and the bioactive CpG 1826 induced the generation the antigen-specific CD8$^+$ T-cell population that was maintained for at least 2 months.

The TSYKFESV-specific CD8$^+$ T-cell population in antiviral protection

Further, we investigated whether the generated TSYKFESV-specific CD8$^+$ T-cell population participated in the protection against ECTV challenge. C57BL/6 mice were immunized as described earlier using systemic or mucosal model. Then 10 days (the acute phase of immune response) or 60 days (the memory phase of immune response) post second immunization, mice were ip challenged with 10^5 PFU of ECTV-Mos/mouse. Specificity and functionality of the generated CD8$^+$ T cells were assessed by B8R-pentamer labeling and intracellular IFN-γ staining, respectively, on the second and fourth day post-challenge in comparison to the appropriate groups of unchallenged animals (day 0). Additionally, the viral load was measured in the spleen tissue (PFU/g). The major points are to be noted.

First, splenic and DLNs responses evaluated on the second and fourth day post ip ECTV challenge revealed almost 2-fold decrease of CD8$^+$/IFN-γ^+ and CD8$^+$/B8R-pentamer$^+$ percentage in spleen and DLNs of C57BL/6 mice during the acute response to mucosal immunization with the TSYKFESV peptide and the bioactive CpG 1826 compared to the unchallenged group (Fig. 3A and B). These results were reproducible and now we can speculate that during ECTV challenge the generated TSYKFESV-specific CD8$^+$ T cells infiltrated the peritoneal cavity and the abdominal fat pads but to confirm that further evaluation is required. Additionally, small but not significant, decrease of the same T-cell population was observed in spleen and DLNs of C57BL/6 mice immunized systemically with the TSYKFESV peptide and the bioactive CpG 1826 and ip challenged during the acute phase of immune response (Fig. 3A and B).

C57BL/6 mice challenged with ECTV during the memory phase generated responses approximate or equal to their appropriate controls irrespectively of the immunization route, indicating no change in the frequency of CD8$^+$/INF-γ^+/B8R-pentamer$^+$ T cells. Furthermore, slight increase of the TSYKFESV specific CD8$^+$ T cells on the second and fourth day post ECTV ip challenge was noted during memory phase of immune response, in contrast to the post challenge results obtained in the acute phase of the immune response (Fig. 3C and D). Moreover, immunofluorescence analysis performed on the freshly isolated CD8$^+$ T cells from spleen of mice that were stimulated either systemically or mucosally with the TSYKFESV peptide and the bioactive CpG 1826, then collected on the second day post ECTV challenge, revealed cross-recognition between the VACV immunodominant epitope-specific CD8$^+$ T cells and ECTV-infected cells (Fig. 4C).

Second, the presence of the CD8$^+$/IFN-γ^+ and CD8$^+$/B8R-pentamer$^+$ T-cell populations was detected during the acute and

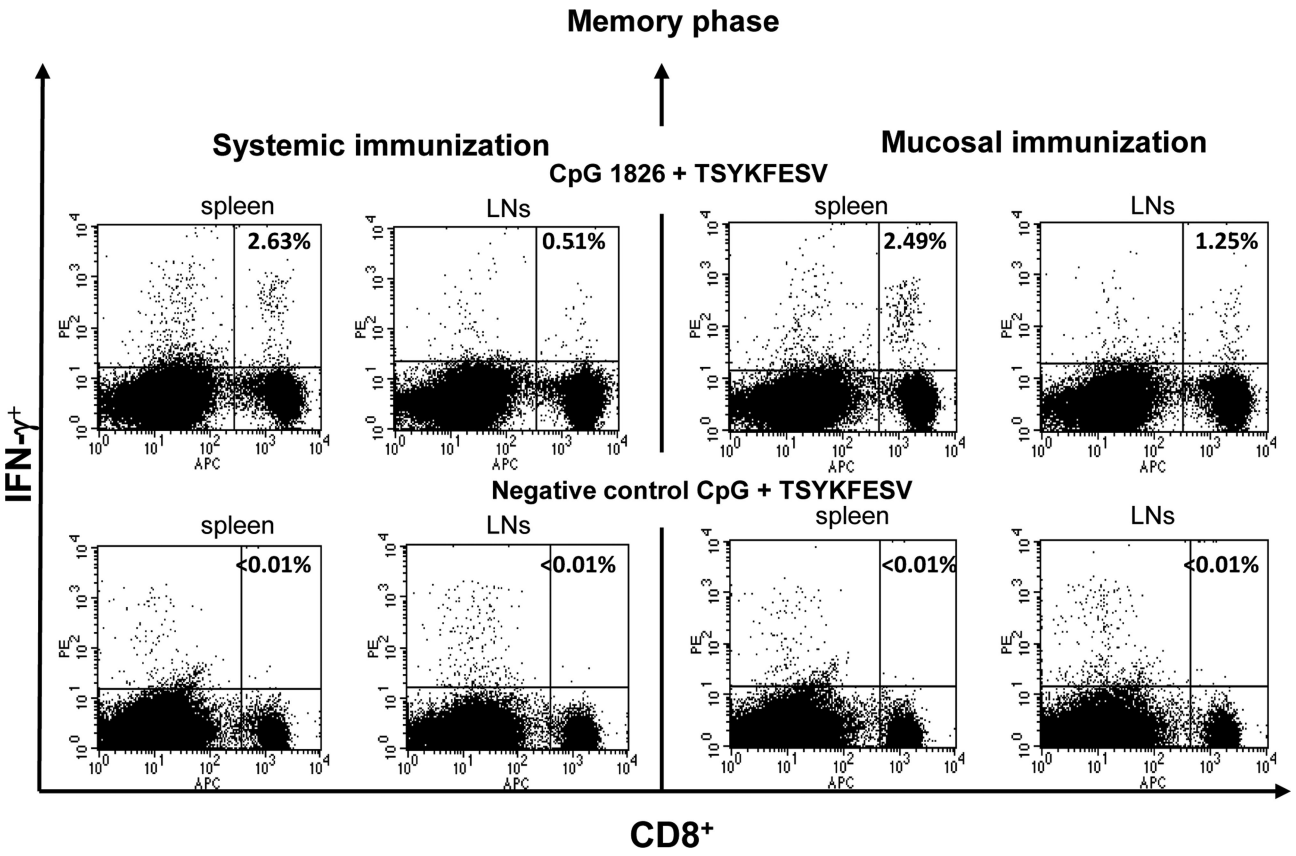

Figure 2. Generation of IFN-γ producing CD8$^+$ T cells in spleen and DLNs of C57BL/6 mice as a result of systemic or mucosal immunization with the TSYKFESV peptide and the bioactive CpG 1826—the memory phase of immune response. C57BL/6 mice were immunized twice on day 0 and 10 either systemically (fp) or mucosally (in) with the immunodominant TSYKFESV petide and either the bioactive CpG 1826 or the negative control CpG. Sixty days post second immunization DLNs and spleen were collected. Obtained cells were stimulated with 1 μg of the TSYKFESV peptide/10^6 cells and 50 U of rIL-2 in the presence of 1μg of brefeldin A for 5 h at 37°C in the presence of 5% CO_2. Subsequently, the CD8 T-cell functionality was assessed by intracellular IFN-γ staining. Flow cytometry analysis in each experimental group was performed on cells isolated from the individual spleen and pooled DLNs. The representative graphs show the CD8$^+$/IFN-γ^+ T-cell populations (upper right quadrant) isolated from C57BL/6 mice during the memory phase of immune response. The numbers within analyzed quadrants represent the percentage of positive cells for a given marker within the gate for lymphocyte population as defined by FSC and SSC parameters.

memory phase of the immune response in spleen of mice immunized with the TSYKFESV peptide and the negative control CpG using either systemic or mucosal route then challenged with ECTV-Mos (Fig. 3C and D).

We provide the evidence that generated, with the immunogen derived from VACV, CD8$^+$/IFN-γ^+/B8R-pentamer$^+$ T cells may influence and control ECTV infection. The viral load in mice spleen was assessed on the second and fourth day post ip challenge. A high level of inhibition of ECTV replication was noted in animals immunized either systemically or mucosally with the TSYKFESV peptide and the bioactive CpG 1826 during the acute phase of the immune response; however, the decrease of virus replication was more remarkable in mice immunized systemically (Table 2). Obtained data qualitatively correlated with the percentages of the generated CD8$^+$/IFN-γ^+/B8R-pentamer$^+$ T cells in those experimental groups. Whereas immunization, either systemic or mucosal, with the TSYKFESV peptide and the bioactive CpG 1826 provided a potent control of ECTV replication during the acute phase of the immune response, in the memory phase animals were less resistant to the ECTV challenge. Nevertheless, there was a significant difference concerning ECTV titers between mice immunized either systemically or mucosally with the TSYKFESV peptide and the bioactive CpG 1826 and animals stimulated with the immunogen and the negative control CpG. Moreover, the immunity induced intranasally persisted since the titer of the reisolated ECTV was lower than the titer of the virus reisolated from systemically stimulated animals. The high ECTV load in all groups correlated with the low percentage of the TSYKFESV-specific CD8$^+$ T cells during the memory phase (Table 2, Fig 3D).

Both the systemic and mucosal immunizations with the TSYKFESV epitope derived from VACV and the bioactive CpG 1826 were responsible for generation of antigen-specific CD8$^+$ T-cell populations. It is highly likely that the preexisting immunodominant peptide-specific CD8$^+$ T cells largely contributed to the inhibition of ECTV replication.

Evaluation of the DCs involvement in the induction of the protective systemic and mucosal immunity

The obtained data showed that the systemic route of immunization induced more effective immunity when evaluated during the acute phase of immune response. Since the assessment during the memory phase presented that both methods of stimulation rendered comparable protective immunity, our goal was to explain the early delay of the immune response induction when mucosal route was applied.

C57BL/6 mice were immunized once, either systemically or mucosally, as described earlier. DLNs (popliteal and inguinal –

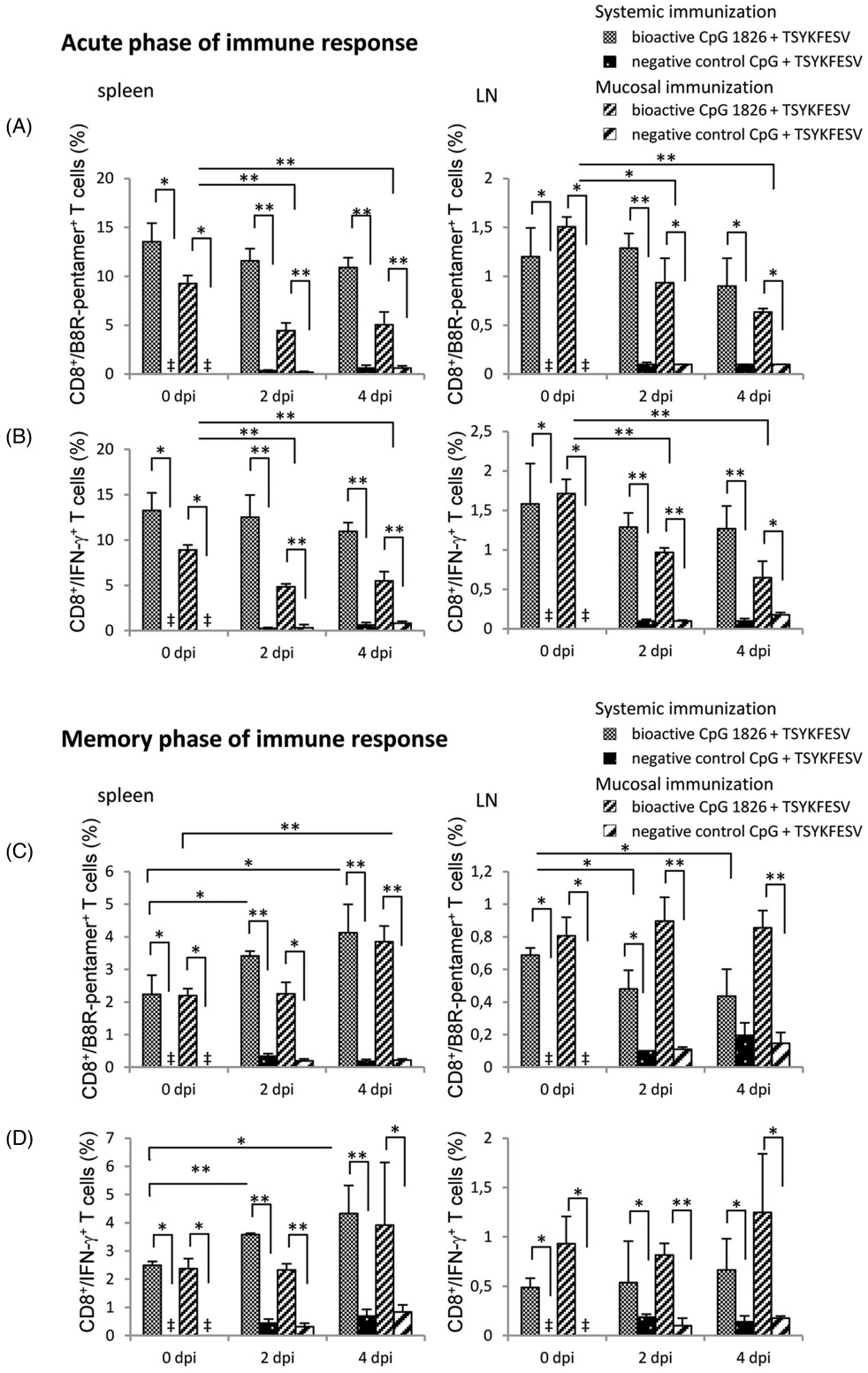

Figure 3. The TSYKFESV-specific $CD8^+$ T cells participate in the protective immunity against ectromelia virus challenge. C57BL/6 mice were immunized twice on day 0 and 10 either systemically (fp) or mucosally (in) with the immunodominant TSYKFESV peptide combined with either the bioactive CpG 1826 or the negative control CpG. Ten days (the acute phase of immune response; **A** and **B**) or sixty days (the memory phase of the immune response; **C** and **D**) post second immunization, mice were ip challenged with 10^5 PFU of ECTV-Mos/mouse. The specificity (A and C) and functionality (B and D) of the generated $CD8^+$ T cells were assessed by B8R-pentamer labeling and intracellular IFN-γ staining, respectively, on the second and fourth day post-challenge in all experimental groups, in comparison to the appropriate groups of unchallenged animals (day 0). The analysis was performed on cells isolated from the individual spleen and pooled DLNs in each experimental group. Data are expressed as mean ± SD from the three or four independent experiments with n = 4/group where $P \leq 0.01$ (double asterisks) was considered as highly statistically significant; and $P \leq 0.05$ (*) was considered as statistically significant. Double dagger – $CD8^+$/B8R-pentamer$^+$ T cells or $CD8^+$/IFN-γ^+ T cells were not detected in the evaluated groups.

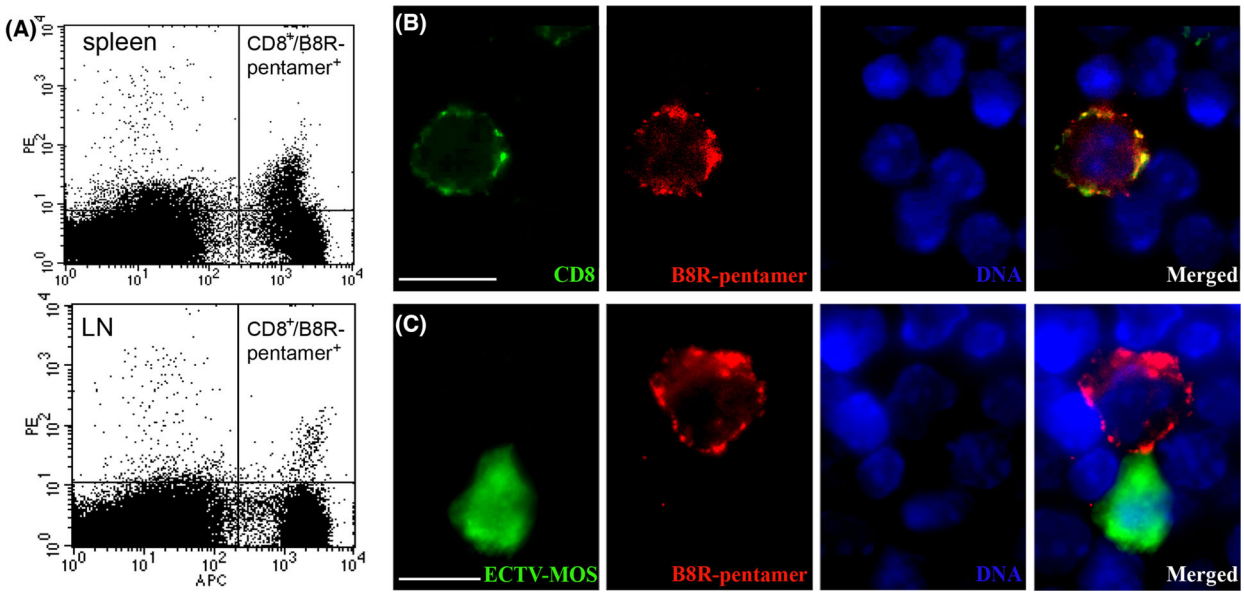

Figure 4. The involvement of CD8$^+$ T cells, generated with the VACV immunodominant epitope and the bioactive CpG 1826, in recognition of the ECTV infected cells. C57BL/6 mice were immunized twice, on day 0 and 10, either systemically (fp) or mucosally (in) with the immunodominant TSYKFESV peptide and either the bioactive CpG 1826 or the negative control CpG. Then, 10 days or 60 days post second immunization mice were ip challenged with 10^5PFU of ECTV-Mos/mouse. Animals were sacrificed on the second and fourth day post-challenge and DLNs and spleen were collected. Subsequently, the involvement of antigen specific CD8$^+$ T cells in protective immunity was assessed by B8R-pentamer labeling and flow cytometer and immunofluorescence analysis was performed. (**A**) The representative graphs show the CD8$^+$/B8R-pentamer$^+$ T-cell population (upper-right quadrants), isolated from spleen and DLNs of C57BL/6 mice immunized with the bioactive CpG 1826 and the immunodominant TSYKFESV peptide, then ip challenged with ECTV. Isolated cells were labeled with anti CD8-FITC mAb and B8R-pentamer (MHC class I/TSYKFESV)-PE. (**B**) Immunofluorescence analysis of CD8$^+$ T cells isolated from C57BL/6 mice immunized twice with the immunodominant TSYKFESV peptide and the bioactive CpG 1826, then challenged with ECTV. Freshly isolated splenocytes were labeled with anti CD8-FITC mAb and B8R-pentamer (MHC class I/TSYKFESV)-PE. Scale bar = 8 μm. (**C**) The image of CD8$^+$ T cells generated with the VACV immunodominant epitope and the bioactive CpG 1826 attacking ECTV infected splenocytes labeled with pAb anti ECTV conjugated with FITC. Analysis was performed on splenocytes freshly isolated from C57BL/6 mice immunized twice with the immunodominant TSYKFESV peptide and the bioactive CpG 1826 and then infected with ECTV-Mos. Scale bar = 8 μm.

Table 2. The influence of the preexisting TSYKFESV-specific CD8$^+$ T cells on ECTV replication in challenged C57BL/6 mice—the acute and memory phase of immune response.

	Immunogen	Time post-challenge (days)	
		2 (1×10^4 PFU/g)	4 (1×10^4 PFU/g)
Acute phase	CpG 1826+TSYKFESV (fp)	0.001 ± 0.001[a]	0.087 ± 0.015
	CpG 1826+TSYKFESV (in)	0.052 ± 0.011	0.97 ± 0.103
	Negative control CpG +TSYKFESV (fp/in)	72.25 ± 12.04	393 ± 82.87
Memory phase	CpG 1826+TSYKFESV (fp)	5.23 ± 0.829	48.75 ± 4.428
	CpG 1826+TSYKFESV (in)	4.42 ± 0.540	25,43 ± 5.673
	Negative control CpG +TSYKFESV (fp/in)	74.70 ± 7.172	506.5 ± 143.9

C57BL/6 mice were immunized twice on day 0 and 10 either systemically or mucosally with the immunodominant TSYKFESV peptide combined with either the bioactive CpG 1826 or the negative control CpG. Then 10 days (the acute phase of immune response) or 60 days (the memory phase of immune response) post second immunization, mice were ip challenged with 10^5 PFU of ECTV-Mos/mouse. On the designated day post-challenge, mice were sacrificed and viral titers were assessed in the spleen homogenates by the plaque assay on Vero cells, measured in PFU/g of isolated tissue. Data are the results of the three independent experiments with n = 4/experiment. [a]values represent mean '±' SD.

in systemic model, cervical – in mucosal model), were collected 18 h post-immunization to obtain pure populations of pDCs and CD11c$^+$DCs. Our pilot studies indicated no significant difference in the level of *IL-12* gene transcript between cell populations isolated from mice immunized with the bioactive CpG 1826 combined with the TSYKFESV peptide regardless of the route of immunization. However, the 3-fold increase of this gene expression was observed in comparison to the results obtained in the same DCs populations isolated from control groups (unpublished data). We quantitatively analyzed mRNA expression of *IL-10*, the cytokine responsible for regulation/silencing immune response and the β subunit of *IL-10* receptor (*IL-10R*β). Analysis performed on cells isolated during the early phase of stimulation showed that pDCs from mice immunized intranasally with the TSYKFESV peptide and the bioactive CpG 1826, expressed significantly ($P \leq 0.05$) higher level of *IL-10* and *IL-10R*β mRNA transcripts in comparison to the same cell population obtained from mice immunized systemically with the TSYKFESV peptide and the bioactive CpG 1826 (Fig. 5). There were no statistically significant differences in mRNA expression of *IL-10* and *IL-10R*β between CD11c$^+$DCs populations isolated from those experimental groups. Irrespectively of the immunization model the level of *IL-10* and *IL-10R*β gene expression was significantly increased in pDCs and CD11c$^+$DCs populations isolated from control groups (Fig. 5). The increased level of *IL-10* and *IL-10R*β mRNA expression in pDCs in the mucosal model shortly after immunization with the TSYKFESV peptide and the bioactive CpG 1826 may explain the differences in the percentage of the generated TSYKFESV-specific CD8$^+$ T cells during the acute phase of the immune response. These data point at the involvement of pDCs

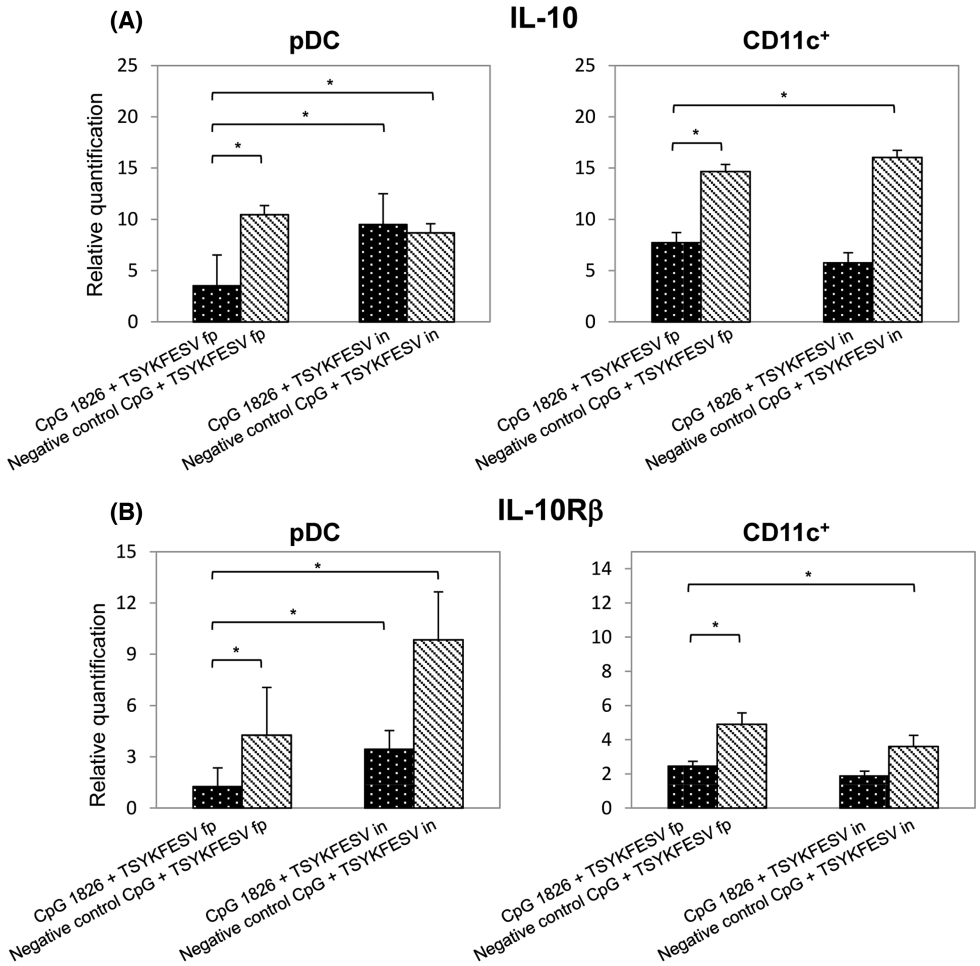

Figure 5. The increased level of *IL-10* and *IL-10Rβ* gene expression in pDCs in mucosal model, influences the development of the adaptive immune response. C57BL/6 mice were immunized either systemically (fd) or mucosally (in) with a single dose of the immunodominant TSYKFESV peptide and either the bioactive CpG 1826 or the negative control CpG. 18 h post-immunization pure populations of pDCs and CD11c$^+$DCs were obtained from DLNs for the total RNA extraction and qPCR analysis was performed as described in the section 'Materials and Methods' using primer sets given in Table 1. (**A**) Analysis of *IL-10* gene expression in pDCs and CD11c$^+$DCs 18 h post either systemic or mucosal immunization. (**B**) Analysis of *IL-10Rβ* gene expression in pDCs and CD11c$^+$DCs 18 h post either systemic or mucosal immunization. Gene expression was determined in each sample using the three independent technical replicates. Bars represent standard errors calculated from three replicates of each experiment. Data are expressed as the mean '±'SD; Asterisk—significantly different values obtained in mice immunized with the immunodominant TSYKFESV peptide and the bioactive CpG 1826 in systemic model compared with values obtained in mice immunized with the immunodominant TSYKFESV peptide and the bioactive CpG 1826 in mucosal model and in mice stimulated with the immunodominant TSYKFESV peptide and the negative control CpG, both systemic and mucosal models; $P \leq 0.05$.

population as a one of the possible key mechanisms responsible for delaying, for a short period of time, the adaptive immune response during the acute phase when the mucosal model was used.

DISCUSSION

Despite the fact that smallpox was eradicated more than 40 years ago, the threat to use poxviruses as the agents of bioterrorism still remains. Moreover, there are emerging zoonoses that can cause variola-like diseases in humans due to cessation of the worldwide vaccination program against VARV in humans (Enserink 2003; Vorou, Papavassiliou and Pierroutsakos 2008; Ninove *et al.* 2009; Quixabeira-Santos *et al.* 2011). The approach for designing a new vaccine against smallpox is required, since the only available vaccine is based on live VACV (Jacobs *et al.* 2009). Subunit vaccines seem to be a good target and in this report we present a potential step to achieve this goal. The present study evaluated the efficacy of the systemic and mucosal immunization with the immunodominant TSYKFESV peptide (VACV B8R$_{20\text{-}27}$) combined with TLR9 agonist – CpG ODNs, as well as functionality of the generated TSYKFESV specific CD8$^+$ T-cell population during heterologous ECTV challenge.

We used CpG ODNs since they have demonstrated potency as the adjuvant to protein and peptide subunit vaccines in humans and animals. Furthermore, they are responsible for the polarization of the immune response toward Th1 cytokine profile (Gierynska *et al.* 2002; Cooper *et al.* 2005; Belyakov *et al.* 2006; Xiao *et al.* 2007; Bode *et al.* 2011) and it has been shown that Th1 cytokine profile is crucial for the resistance and recovery from primary ECTV challenge in the natural host (Chaudhri *et al.* 2004; Sakala *et al.* 2007). Whereas two immunizations performed 10 days apart generated the population of the antigen specific (as measured by ICG and pentamer staining) CD8$^+$ T cells, the real question was, whether the induced T cell-dependent immunity could be involved in protection against heterologous

ECTV challenge in C57BL/6 mice, and how long this response would be maintained in the host. Our results demonstrate that CpG ODNs/peptide administration does induce $CD8^+$ T-cell response against the early immunodominant epitope irrespective of the route of immunization. However, the systemic model was more effective during the acute phase of the immune response. The generated T-cell-dependent immune response was maintained for at least 2 months, although, the frequency of antigen-specific cells decreased over time. Those data support the work of other groups using different systems in CpG ODNs approach (Vabulas *et al.* 2000; Gierynska *et al.* 2002; Bode *et al.* 2011) as well as the results concerning the immune response mounted against the immunodominant TSYKFESV epitope (Tscharke *et al.* 2005; Mathurin *et al.* 2009; Moutafasi *et al.* 2009, Remakus *et al.* 2012).

The early events of the immune response stimulation could be critical for the time delay in the evoking of the adaptive immune response when mucosal model was applied. One of the factors influencing development of the adaptive immune response is IL-10, the cytokine that is crucial for maintaining tolerance in the respiratory mucosa (de Heer *et al.* 2004; Andreev *et al.* 2012; Lambrecht 2012). The detection of the increased level of *IL-10* and *IL-10Rβ* gene expression at the mRNA level in pDCs population isolated from mice shortly after intranasal immunization with the TSYKFESV peptide and the bioactive CpG 1826 may be the explanation of the slight delay in the development of the immune response during the acute phase in mucosal model. Our results are in agreement with other studies that indicate transient increase of *IL-10* gene expression (detection only 24–72 h post-stimulation), responsible for the tolerance induction after exposure to antigen (Akbari, DeKruyff and Umetsu 2001). The other factor that can influence the outcome of the immune response, not addressed in this paper, is the expression of TLRs on DCs that seemed to be organ specific. The study comparing the expression of TLR4 and TLR9 on lung and spleen DCs revealed that whereas lung DCs expressed higher levels of TLR4 but only very low levels of TLR9, spleen DCs had the opposite TLRs pattern (Chen *et al.* 2006). The major goal of vaccination is the induction of the long-lived mucosal immunity that can effectively block pathogen replication. When analysis was performed 2 months after immunization with the TSYKFESV peptide and the bioactive CpG 1826, slight differences in the level of antigen-specific $CD8^+$ T-cell population favored mucosal model. Our data support the results obtained by Belyakov *et al.*, where they point at the intranasal immunization with the CpG ODNs and modified vaccinia Ankara, as a more effective method of inducing $CD8^+$ T-cell immunity and protection against lethal VACV Western Reserve strain than intramuscular immunization (Belyakov *et al.* 2006).

C57BL/6 mice are considered resistant to the development of the lethal form of ectromelia (Fenner and Buller 1997). However, the most invasive, ip, challenge route or high dose of the infectious agent is able to overcome mice resistance. These conditions were chosen to assess the involvement of the TSYKFESV specific $CD8^+$ T cells as well as to minimize the influence of the early anti-viral immune response mechanisms which C57BL/6 resistance depends on (IFN type I, II, III, NK cells, $\gamma\delta$T cells, macrophages) (Fenner and Buller 1997; Chaudhri *et al.* 2004; Esteban and Buller 2005; Gierynska *et al.* 2009; Remakus *et al.* 2012). There is a strong indication that the preexisting TSYKFESV-specific $CD8^+$ T cells were the major source of IFN-γ during the early phase of infection and were likely to limit ECTV replication, thus preventing viral dissemination. While cytotoxicity of this population was not evaluated in our studies, but the IFN-γ production by antigen-specific $CD8^+$ T cells and cytotoxicity is strongly correlated (Kagi *et al.* 1994; Guidotti *et al.* 1999; Remakus *et al.* 2012). Moreover, there was a direct, specific recognition of heterologously, ECTV infected cells by the TSYKFESV-specific $CD8^+$ T cells. These data are in agreement with the work of Remakus *et al.*, and although different immunization model (TSYKFESV-induced DCs) and infection route was used, both studies point at the memory $CD8^+$ T cells of the single specificity as a tool responsible for the protective immunity (Remakus *et al.* 2012) or, as we showed, reduction of ECTV replication. This level of protection waned in time and it was correlated with the decreased number of the TSYKFESV-specific $CD8^+$ T cells. But even 2 months after immunization the differences concerning the viral load in spleen were evident between groups of mice immunized with the bioactive CpG 1826 and the TSYKFESV peptide (systemic and mucosal models), and control groups. These results open new pathways to solve the problem with the decreasing level of cytotoxic, TSYKFESV-specific $CD8^+$ T cells. Studies carried out by Toka *et al.* (2005) clearly show that administration of the exogenous IL-15 or anti-CD40 mAb during memory phase can rescue $CD8^+$ T-cell population generated during immunization with MHC I restricted epitope and TLR9 agonist. The approach of using the VACV CD8 epitope combined with the bioactive CpG ODNs resulted in the induction of cytotoxic $CD8^+$ T cells crucial for the resistance and recovery from the primary ECTV challenge. However, the studies of Panchanathan, Chaudhri and Karupiah (2005) clearly showed the importance of the humoral immunity during orthopoxviruses infection and that IFN-γ was not required for recovery from a secondary poxvirus infection in mice deficient in type I and II IFN function. These animals were able to control virus effectively due to the ability to generate neutralizing antibodies. The studies of Xiao *et al.* (2007) focused on designing a protein-based vaccine confirmed the value of humoral immune response. On the other hand, the role of $CD8^+$ T-cell-dependent immunity mediated by IFN-γ was recently presented in the model of respiratory infection with VACV Western Reserve strain (Goulding *et al.* 2012, 2014) where IFN-γ signaling was responsible for lung pathology reduction, virus dissemination inhibition and early clearance of the virus. Also the model with the recombinant VACV expressing IFN-γ used for post-exposure protection against VACV and ECTV emphasized the role of this cytokine (Holechek *et al.* 2013) and the studies of Xu *et al.* (2007), clearly showed that not only antibodies but also recall responses by long-lived memory $CD8^+$ T cells could prevent clinical mousepox. Combination of proteins and the immunodominant $CD8^+$ T-cell epitopes with proper adjuvants could be an initial strategy to provide the baseline immunity against orthopoxvirus infections that allows the development of effective CTLs and neutralizing Ab. With an increasingly immunocompromised human population due to the cancer treatment, anti-inflammatory treatment, organ transplantations, infection with HIV, or those with atopic skin disorders, every attempt to induce antiviral immunity with a safe vaccine is essential.

In conclusion, the concept of using well-selected $CD8^+$ T cells specific epitope and TLR agonist in generation of the adaptive immune response to related, yet heterologous virus within the *Poxviridae* family, was endorsed under experimental conditions and hold promise for future design of subunit vaccine against smallpox, since the generated TSYKFESV-specific $CD8^+$ T cells definitely influenced ECTV replication during challenge. This approach may be of benefit when a novel smallpox vaccine is to be developed, since despite the fact that this disease was eradicated more than 40 years ago, the use of orthopoxviruses by

bioterrorists remains a potential and growing threat. However, further manipulations are required to maintain the proper level of fully functional induced CD8 T-cell immunity.

ACKNOWLEDGEMENTS

Authors thank the Staff of collaborating laboratories for their support. We are grateful to Professor Anna Winnicka from Flow Cytometry laboratory, Division of Animal Pathophysiology, Faculty of Veterinary Medicine, for her valuable and expert assistance. We are indebted to Professor Barry T. Rouse from University of Tennessee, Knoxville, USA, and we express our thanks for the helpful discussion and critical reading of this manuscript.

FUNDING

This work was supported by grant of National Science Center, Poland, no NN308 07 4339.

Conflict of interest. None declared.

REFERENCES

Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol* 2001;**2**:725–31.

Alcami A, Hill B, Koszinowski U. Viral interference with the host immune response. In: *Topley and Wilson's Microbiology and Microbial Infections*. John Wiley and Sons, 2010, 617–44. DOI:10.1002/9780470688618.taw0112

Andreev K, Graser A, Maier A, *et al*. Therapeutical measures to control airway tolerance in asthma and lung cancer. *Front Immunol* 2012;**3**:216, DOI: 10.3389/fimmu.2012.00216.

Belyakov IM, Isakov D, Zhu Q *et al*. Enhancement of $CD8^+$ T cell immunity in the lung by CpG oligodeoxynucleotides increases protective efficacy of a modified vaccinia Ankara vaccine against lethal poxvirus infection even in CD4-deficient host. *J Immunol* 2006;**177**:6336–43.

Bode C, Zhao G, Steinhagen F, *et al*. CpG DNA as a vaccine adjuvant. *Expert Rev Vaccine* 2011;**10**:499–511.

Chaudhri G, Panchanathan V, Buller RM, *et al*. Polarized type cytokine response and cell-mediated immunity determine genetic resistance to mousepox. *P Natl Acad Sci USA* 2004;**101**:9057–62.

Chen L, Arora M, Yarlagadda M, *et al*. Distinct responses of lung and spleen dendritic cells to the TLR9 agonist CpG oligodeoxynucleotide. *J Immunol* 2006;**177**:2373–83.

Chung CYJ, Ysebaert D, Berneman ZN, *et al*. Dendritic cells: cellular mediators for immunological tolerance. *Clin Dev Immunol* 2013;**2013**:972865. http://dx.doi.org/10.1155/2013/972865. (19 October 2015, date last accessed).

Cooper CL, Davis HL, Angel JB, *et al*. CpG 7909 adjuvants improves hepatitis B virus vaccine seroprotection in antiretroviral-treated HIV-infected adults. *AIDS* 2005;**19**:1473–9.

de Heer HJ, Hammad H, Soullié T, *et al*. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J Exp Med* 2004;**200**:89–98.

Enserink M. U.S. monkeypox outbreak traced to Wisconsin pet dealer. *Science* 2003;**300**:1639.

Enserink M. Biodefense. Smallpox vaccines: looking beyond the next generation. *Science* 2004;**304**:809.

Esteban DJ, Buller RML. Ectromelia virus: the causative agent of mousepox. *J Gen Virol* 2005;**86**(Pt10):2645–59.

Fenner FR, Buller RML. Mousepox. In: Nathanson N (ed.). *Viral Pathogenesis*. Philadelphia: Lippincott Raven, 1997, 535–53.

Fulginiti VA, Papier A, Lane JM, *et al*. Smallpox vaccination: a review, part II. Adverse events. *Clin Infect Dis* 2003;**37**:251–71.

Gallichan WS, Woolstencroft RN, Guarasci T, *et al*. Intranasal immunization with CpG oligodeoxynucleotides as an adjuvant dramatically increases IgA and protection against herpes simplex virus-2 in the genital tract. *J Immunol* 2001;**166**:3451–7.

Garcel A, Fauquette W, Dehouck M-P, *et al*. Vaccinia virus-induced smallpox postvaccinal encephalitis in case of blood-brain barrier damage. *Vaccine* 2012;**30**:1397–405.

Gierynska M, Kumaraguru U, Eo S-K, *et al*. Induction of CD8 T cells specific, systemic and mucosal immunity against HSV with CpG – peptide complexes. *J Virol* 2002;**76**:6568–76.

Gierynska M, Pawlak E, Schollenberger A, *et al*. Dendritic epidermal T cells—their role in the early phase of infection with ectromelia virus. *Post Hig Med Dosw* 2009;**63**:369–76.

Goulding J, Abboud G, Tahiliani V, *et al*. CD8 T cells use IFN-g to protect against the lethal effects of a respiratory poxvirus infection. *J Virol* 2014;**192**:5415–25.

Goulding J, Bogue R, Tahiliani V, *et al*. CD8 T cells are essential for recovery from a respiratory vaccinia virus infection. *J Immunol* 2012;**189**:2432–40.

Guidotti LG, Borrow P, Brown A, *et al*. Noncytopathic clearance of lymphocytic choriomeningitis virus from the hepatocyte. *J Exp Med* 1999;**189**:1555–64.

Halsell JS, Riddle JR, Atwood JE, *et al*. Myopericarditis following smallpox vaccination among vaccinia-naive US military personnel. *JAMA* 2003;**289**:3283–9.

Henderson DA. The looming threat of the bioterrorism. *Science* 1999;**283**:1279–82.

Holechek S, Denzler K, Heck MC, *et al*. Use of a recombinant vaccinia virus expressing interferon gamma for post-exposure protection against vaccinia and ectromelia viruses. *PLoS One* 2013;**8**:e77879.

Jacobs BL, Langland JO, Kibler KV, *et al*. Vaccinia virus vaccines: past, present and future. *Antivir Res* 2009;**84**:1–13.

Jenner E. *An Enquiry Into the Causes and Effects of the Variolae Vaccinae, a Disease Discovered in Some of the Western Counties of England, Particularly Gloucestershire, and Known by the Name of the Cow Pox*, 1st edn. By: London, England, 1798.

Kagi D, Ledermann B, Burki K, *et al*. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* 1994;**369**:31–7.

Klinman DM. Immunotherapeutic uses of CpG oligodeoxnucleotides. *Nat Rev Immunol* 2004;**4**:249–58.

Krieg AM. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol* 2002;**20**:709–60.

Lambrecht BN, Hammad H. Lung dendritic cells in respiratory viral infection and asthma: from protection to immunopathology. *Ann Rev Immunol* 2012;**30**:243–70.

Lewis KL, Reizis B. Dendritic cells: arbiters of immunity and immunological tolerance. *Cold Spring Harb Perspect Biol* 2012;**4**:a007401.

McFadden G. Poxvirus tropism. *Nat Rev Microbiol* 2005;**3**:201–13.

Mathurin KS, Martens GW, Kornfeld H, *et al*. CD4 T-cell-mediated heterologous immunity between mycobacteria and poxviruses. *J Virol* 2009;**83**:3528–39.

Mendez S, Tabbara K, Belkaid Y, *et al*. Coinjection with CpG-containing immunostimulatory oligodeoxynucleotides reduces the pathogenicity of a live vaccine against cutaneous Leishmaniasis but maintains its potency and durability. *Infect Immun* 2003;**71**:5121–9.

Moutafasi M, Peters B, Pasquetto V, *et al*. A consensus epitope prediction approach identifies the breadth of murine T CD8$^+$-cell responses to vaccinia virus. *Nat Biotechol* 2006;**24**: 817–9.

Moutafasi M, Salek-Ardakani S, Croft BP, *et al*. Correlates of protection efficacy induced by vaccinia virus-specific CD8$^+$ T-cell epitopes in the murine intranasal challenge model. *Eur J Immunol* 2009;**39**:717–22.

Naik AH, Park H-Y, Metcalf D, *et al*. Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived *in vitro* and *in vivo*. *Nat Immunol* 2007;**8**: 1217–26.

Ninove L, Domart Y, Vervel C, *et al*. Cowpoxvirus transmission from pet rats to humans, France. *Emerg Infect Dis* 2009;**15**: 781–4.

Nuara AA, Bai H, Chen N, *et al*. The unique C termini of orthopoxvirus gamma interferon binding proteins are essential for ligand binding. *J Virol* 2006;**80**:10675–82.

Onai N, Obata-Onai A, Schmid MA, *et al*. Identification of clonogenic common Flt3$^+$M-CSFR$^+$ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. *Nat Immunol* 2007;**8**:1207–16.

Panchanathan V, Chaudhri G, Karupiah G. Interferon function is not required for recovery from a secondary poxvirus infection. *P Natl Acad Sci USA* 2005;**102**:12921–6.

Poland GA, Grabenstein JD, Neff JM. The US smallpox vaccination program: a review of a large modern era smallpox vaccination implementation program. *Vaccine* 2005;**23**:2078–81.

Quixabeira-Santos JC, Medaglia ML, Pescador CA, *et al*. Animal movement and establishment of vaccinia virus contagalo strain in Amazon Biome, Brazil. *Emerg Infect Dis* 2011;**17**: 726–9.

Remakus S, Rubio D, Ma X, *et al*. Memory CD8$^+$ T cells specific for a single immunodominant or subdominant determinant induced be peptide-dendritic cell immunization protect from an acute lethal viral disease. *J Virol* 2012;**86**:9748–59.

Rosenthal SR, Merchlinsky M, Kleppinger C, *et al*. Developing new smallpox vaccines. *Emerg Infect Dis* 2001;**7**:920–6.

Sakala IG, Chaudhri, Buller RML G, *et al*. Poxvirus-encoded gamma interferon binding protein dampens the host immune response to infection. *J Virol* 2007;**81**:3346–53.

Schatzmayr HG, Costa, RVC, Gonçalves MCR, *et al*. Human and animal infections by vaccinia-like viruses in the state of Rio de Janeiro: a novel expanding zoonoses. *Vaccine* 2011;**29**(suppl.4):D65–9.

Smith VP, Alcami A. Inhibition of interferons by ectromelia virus. *J Virol* 2002;**76**:1124–34.

Steinman RM, Banchereau J. Taking dendritic cells into medicine. *Nature* 2007;**449**:419–26.

Toka FN, Gierynska M, Suvas S, *et al*. Rescue memory CD8$^+$ T cell reactivity in peptide/TLR9 ligand immunization by codelivery of cytokines or CD40 ligation. *Virology* 2005;**331**:151–8.

Tscharke DC, Karupiah G, Zhou J, *et al*. Identification of poxviruses CD8$^+$ T cell determinants to enable rational design and characterization of smallpox vaccines. *J Exp Med* 2005;**201**:95–104.

Tscharke DC, Woo WP, Sakala IG, *et al*. Poxvirus CD8$^+$ T-cell determinants and cross-reactivity in BALB/c mice. *J Virol* 2006;**80**:6318–23.

Vabulas RM, Pircher H, Lipford GB, *et al*. CpG-DNA activates *in vivo* T cell epitope presenting dendritic cells to trigger protective antiviral cytotoxic T cell responses. *J Immunol* 2000;**164**:2372–8.

Vorou RM, Papavassiliou VG, Pierroutsakos IN. Cowpox virus infection: an emerging health threat. *Curr Opin Infect Dis* 2008;**21**:153–6.

Welsh RM, Selin LK. No one is naïve: the significance of heterologous T-cell immunity. *Nat Rev Immunol* 2002;**2**: 417–26.

Xiao Y, Aldaz-Carroll L, Ortiz AM, *et al*. A protein – based smallpox vaccine protects mice from vaccinia and ectromelia virus challenges when given as a prime and single boost. *Vaccine* 2007;**25**:1214–24.

Xu RH, Fang M, Klein-Szanto A, *et al*. Memory CD8$^+$ T cells are gatekeepers of lymph node draining the site of viral infection. *P Natl Acad Sci USA* 2007;**104**:10992–7.