Simultaneous infection with gammaherpes and influenza viruses enhances the host immune defense

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Summary. – We have studied the impact of simultaneous infection of mice with murine gammaherpesvirus (MHV) and influenza A virus (IAV) on the immune response and pathogenesis of both infections. After a persistent MHV-68 herpesviral infection had been established, the same mice were super-infected with IAV. Individual parameters of MHV infection (viral DNA detection in organs and blood) and numbers of leukocytes in lungs and spleens were determined. With regard to the assumed reactivation of MHV-68 (mainly in lungs, spleen, thymus and peritoneal exudate cells) we focused our attention on the detection of transcripts, typical either for lytic infection (ORF50) and/or for latency (ORF73). Herpesviral DNA was detected in above mentioned organs in several intervals during the acute phase of IAV co-infection, but the expression of monitored transcripts was lower, i.e. it has decreased. Though the reason for such limited expression during acute influenza superinfection remains unclear, it is unambiguous that lower MHV-68 expression was detected in lungs and peritoneal exudate cells (PECs) from 3rd to 10th day after co-infection with IAV. Furthermore, our study showed that the ongoing gammaherpesvirus latency in co-infected mice affected the number of cytotoxic T-lymphocytes and neutrophils during the acute IAV infection and lowered their deviations from that of non-infected mice. Therefore, we suppose that co-infection with herpes and influenza viruses could be mutually beneficial for the host by promoting its defense against both viruses.

Keywords: murine herpesvirus; influenza A virus; co-infection; latency

Introduction

Most hosts, including humans, are often concurrently infected with multiple pathogens, frequently by herpesviruses. Hosts that are immunologically challenged by one infection often show increased susceptibility to a second infective agent. This has been demonstrated with various groups of pathogens, including parasites, protozoa, bacte-

ria, and viruses (Thakar *et al.*, 2012; Griffiths *et al.*, 2011). On the other hand, interaction between several infectious agents may lead to altered immune responses, leading to heterologous immunity or cross-protective immunity. Several research teams in the last few years investigated the double co-infection, especially its impact on host immunity and overall course of infection (Florescu *et al.*, 2011; Smith *et al.*, 2013). In previous studies it has been shown that mice infected with certain groups of herpesviruses, such as mouse cytomegalovirus and murine gammaherpesvirus (MHV), developed a resistance to intracellular bacterial pathogens such as *Listeria monocytogenes* and *Yersinia pestis*. These data evoke a speculation that under certain conditions harboring herpesviruses may be a survival benefit (due to mechanisms) which tends to be host species-specific and which had been formed by co-

evolution within their hosts during a long period of time (Barton *et al.*, 2007).

Humans become infected with multiple herpesviruses during childhood. After clearance of the acute (stage of) infection, herpesviruses enter a dormant state known as latency. Latency persists for the life of the host and it is presumed to be parasitic, as it leaves the individual at risk for subsequent viral reactivation and diseases (Welsh *et al.*, 2010). Epstein-Barr virus (EBV) is a human gammaherpesvirus with high worldwide prevalence in adults (up to 90%), which is able to establish life-long persistent infection in resting memory B cells in its host. This virus is maintained in a balance between viral homeostasis on one side and antiviral defense of the immune system on the other side. Latent EBV infection is commonly associated with human cancer, in particular, with lymphoid malignancies and/or nasopharyngeal carcinoma (Arfelt *et al.*, 2015; Hopwood and Crawford, 2000; Mutalima *et al.*, 2008; Rickinson and Kieff, 2007). Childhood infection with EBV is often subclinical, while the same infection is frequently symptomatic in adolescents and adults and is manifested as infectious mononucleosis (IM). Some studies confirm that cross-reactive influenza virus-specific CD8^{+} T cells contribute to lymphoproliferation of EBV-associated IM (Clute *et al.*, 2005). However, the cross-reactive cells that dominate this response have low affinity for the virus antigen-expressing cells and cannot control the infection adequately. Despite the apparent advantage of a pre-existing immune repertoire, the net result is immunopathology. This effect may underlie the observation that some infections, such as measles, mumps, chickenpox, and EBV, have more severe consequences in teenagers and young adults than they do in young children (Welsh and Fujinami, 2007; Sharma and Thomas, 2014).

MHV-68 is a natural rodent pathogen that is structurally, biologically, and genetically related to human herpesviruses. Intranasal infection of mice with MHV-68 causes an acute respiratory infection that is rapidly resolved, followed by the establishment of a life-long latency in B-lymphocytes, macrophages and dendritic cells. Because of its relatedness to human gammaherpesviruses, experimental MHV-68 infection provides a natural model for the study of γ-herpesvirus pathology and host immunity *in vivo* (Blaškovič *et al.*, 1980; De Lima *et al.*, 2005; Nash *et al.*, 2001; Kúdelová and Rajčáni, 2007).

The research of double infection is important to clarify the mortality among previously clinically healthy and/or young individuals due to influenza infection, and such studies are highly relevant because most of the population is latently infected with at least one of the two gammaherpesviruses. Understanding how co-infection can alter the possible overwhelming immune response, cytokine storm, inflammatory immunopathology or potential cross-protection and heterologous immune response could be critical for the development of novel strategies for the prevention and treatment of these infections.

Saito *et al.* (2013) demonstrated that MHV-68 latency results in accelerated influenza viral clearance at early time points, leading to lower influenza viral loads and decreased inflammatory pathology in the lungs. Further, they investigated differences in numbers of lung inflammatory cells and suggested the importance of CD8^{+} T cells and the number of neutrophils in antiviral immunity (Saito *et al.*, 2013). Other study provided evidence that latently infected mice had lower levels of IFN-γ in lung homogenates and that MHV-68 viral load in the spleen were unaltered by influenza infection (Hardisty *et al.*, 2013). Recent studies with MHV-68 and lymphocytic choriomeningitis virus (LCMV) demonstrated that ongoing gammaherpesvirus latency affected the number of CD8^{+} T cells during acute LCMV infection (Barton *et al.*, 2014). Other research team found that helminth infection reactivated murine gammaherpesvirus infection *in vivo* (Reese *et al.*, 2014). Yager *et al.* proved that early MHV-68 latency conferred protection against bacterial infection, but it was shown that the effect was transient (Yager *et al.*, 2009).

The aim of the current study is to contribute to the understanding how acute viral infection influences pathogenesis and latency control of persistent infection. Recent research studies hypothesized that MHV-68 latency would provide the host with protective immunity against influenza virus infection (Saito *et al.*, 2013; Hardisty *et al.*, 2013). In this study we did not focus on understanding the mechanism underlying the immunity to the influenza virus, but – here we attempted to decipher whether the acute influenza infection can alter the ongoing latent MHV-68 infection. We assumed that during co-infection, ability of a pathogen to influence the immune response against another pathogen can work both ways, and that an acute respiratory viral infection could modulate many parameters of lytic and/or latent MHV-68 infection. Our results provide evidence that an acute respiratory viral infection impacts the host immune response to a latent gammaherpesviral infection.

Our findings can broaden the knowledge about gammaherpesvirus pathogenesis and enlighten possible risks of double infection that can often occur in reality. In clinical practice latent infection with human herpesviruses EBV and Kaposi´s sarcoma associated herpesvirus, even when undeterminable, may play a hitherto unrecognizable role in affecting the immune response to respiratory pathogens like influenza virus.

Material and Methods

Cells, viruses and mice. Vero cells and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 9% of heat-inactivated fetal bovine serum (FBS),

glutamine, penicillin and streptomycin. MHV-68 stock for *in vitro* and *in vivo* experiments was prepared by virus propagation on NIH 3T3 cells, while titers of infectious viruses were determined by plaque assay using Vero cells. Influenza A/Mississippi/1/85 (H3N2) strain was propagated in the allantoic cavity of embryonated chicken eggs and adapted on Balb/c mice as done previously (Fislová *et al.*, 2009).

Animal experiments. Female 6–8-week-old inbred BALB/c mice were supplied by the Faculty of Veterinary Medicine, Brno, Czech Republic. Mice were intranasally infected with 2×10^4 PFU of MHV-68 per mouse in total inoculum volume of 20 µl, under light anesthesia. Forty-five days after herpesviral infection mice were intranasally infected with human mouse adapted influenza virus A/Mississippi/1/85/M9/E2 (H3N2). For infection a sublethal dose: $0.4\ LD_{50}$ (78.8 PFU/mouse) was used, in volume of 40 µl. Control group of mice received intranasally 20 µl of phosphate buffered saline (PBS). Three experimental groups of 64 animals and one control group of 12 mice were set up (Table 1). Mice were killed by cervical dislocation under anesthesia in six different time points and various organs collected from mice were analyzed. All animal experiments were performed according to the European Union standards and fundamental ethical principles including animal welfare requirements were respected. All experiments were done with the approval of State Veterinary and Food Administration of the Slovak Republic (2937/10-221).

Blood sample analysis. Blood samples were taken from *sinus orbitalis* at different time points post infection for serum preparation and examination of leukocytes. The obtained sera were inactivated for 30 min. at 56°C and stored at -20°C until used in virus neutralization assay. Blood samples were mixed with heparin (final concentration of 2–4 U/ml) and stained with Turck´s solution to determine the total leukocyte count microscopically. Heparinized blood was used also for blood smears, which were stained with May-Grünwald solution (for 10 min.) and Giemsa-Romanowski solution (for 15 min.) and differential blood cell count was evaluated.

Virus-neutralization assay. 2-fold dilutions of inactivated immune sera were incubated with 1000 PFU of MHV-68. After 30 min incubation at 37°C, virus-serum mixtures were inoculated onto Vero cell monolayers. The last dilution of serum that was able to prevent virus infection in cell culture was considered as the titer of virus-neutralization antibodies.

Detection of viral genome and transcripts. Total DNA was extracted from 300 µl of heparinized blood and of organ homogenates using Wizard® genomic DNA purification kit (Promega). Total DNA was used as a template in nested PCR using M7 primers: M7 (forward 1): CTCGAACAACAATCCCACTACA and M7 (reverse 1): CGTAT CCAAAGCAGGGTAGAAA, M7 (forward 2): GCCCAAAA TGGTGAGAGTGTA and M7 (reverse 2): GGTGGGTTCATC TTCCTGATT. Total RNA was extracted from peritoneal exudate cells (PECs), lungs and spleen of MHV-68 infected and herpes + influenza co-infected mice. Lungs and spleen were homogenized using 70 µm nylon cell strainers (BD Falcon), 1 ml TRI Reagent® (Sigma-Aldrich) was added and extraction was carried out according

to manufacturer´s instructions. Complementary DNA was synthesized from total RNA by Revert aid first strand cDNA synthesis kit (Thermo Scientific). Mouse β-actin was used as an internal control, using primers: Act (forward): TCCTCCCTGGAGAAGAGCTA and Act (reverse): ACATCTGCTGGAAGGTGGAC. Thus verified cDNA was used as a template in nested PCR in which transcripts of ORF 50 (immediate-early) and ORF 73 (latent) were analyzed. Sets of gene-specific primers used for nested PCR were as follows: ORF73 (forward 1): TAGTACCTTCTACGCTGCTG and ORF73 (reverse 1): GTAGGTGCTTCAACAAACCA, ORF73 (forward 2): CCAAG GGTAAACAGCTAACTG and ORF73 (reverse 2): CCTTCATCAC CAGTACATGAG, ORF50 (forward 1): CCACCTGATCAAATAT GCCA and ORF50 (reverse 1): TGTGGGTTTCTTGTTTGGAC, ORF50 (forward 2): TGGCATATCCAGAGAAGTTGAG and ORF50 (reverse 2): TGGGAGTAGGTATGTAGCTCTG.

Flow cytometry. Spleens were harvested and mechanically dispersed into a single cell suspension using 70 µm nylon cell strainers, and ammonium chloride lysis buffer was used to lyse erythrocytes. Single cell suspensions of lungs were prepared by dispersing. Lungs were minced in supplemented PBS containing 0.1% type A collagenase (Sigma-Aldrich) for 30 min at 37°C. Cells were fixed on ice for 20 min. with parafolmaldehyd in supplemented versen solution containing 1% fetal bovine serum and stained with the indicated antibodies: rat monoclonal to CD3 epsilon PE (Abcam) or anti-mouse CD3e PE (eBioscience); anti-mouse CD4PerCP – Cy 5.5 (eBioscience); anti-mouse CD8a/Lyt – 2 – FITC (BECKMAN COULTER); anti-mouse CD19 FITC (CD5) (PROIMMUNE) – (CD19 PE-Cy5 (6D5); anti-mouse CD45 PE (eBioscience) and rat anti-mouse Ly-6G FITC (BD Pharmingen) on ice for 20–30 min. Cells were analyzed using Guava easyCyte™ HT system (Millipore) and data generated were analyzed by software InCyte 2.5.
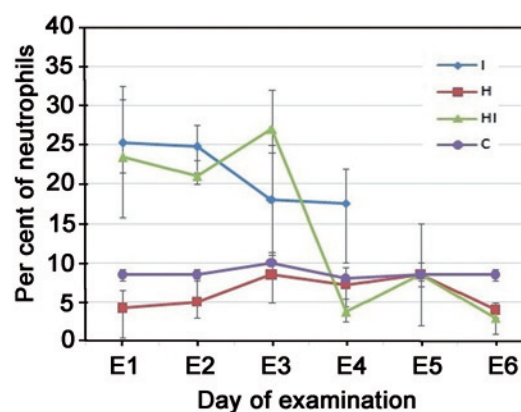

Fig. 1

Percentage of neutrophils from a total number of leukocytes (100%) in peripheral blood of persistently infected mice after co-infection with influenza A virus

The value for each group represents the mean ± standard deviation for four mice. I = IAV infected mice; H = MHV-68 infected mice; HI = IAV and MHV-68 co-infected mice; C = control mice; E1-E6 = days of examination (see Table 1).

Table 1. Time schedule describing days of examination. Four infected mice and 2 control mice per group were used for analysis at each time point

| Day of experiment | Day of examination | Group of mice | | | |
		H MHV-68[*]	I A/Miss[**]	HI MHV-68[*] + A/Miss[**]	C Mock infection[*] (control)
Day 48	E1	=48 dpi	=3 dpi	=48 dpi with MHV-68 =3 dpi with A/Miss	=48 dpm
Day 51	E2	=51 dpi	=6 dpi	=51 dpi with MHV-68 =6 dpi with A/Miss	=51 dpm
Day 55	E3	=55 dpi	=10 dpi	=55 dpi with MHV-68 =10 dpi with A/Miss	=55 dpm
Day 105	E4	=105 dpi	=60 dpi	=105 dpi with MHV-68 =60 dpi with A/Miss	=105 dpm
Day 180	E5	=180 dpi	ND	=180 dpi with MHV-68 =135 dpi with A/Miss	=180 dpm
Day 270	E6	=270 dpi	ND	=270 dpi with MHV-68 =225 dpi with A/Miss	=270 dpm

A/Miss = A Mississippi/1/85 (H3N2); dpm = day post mock-infection; [*]Infected on day 1; [**]Infected on day 45.

Results

Clinical symptoms of co-infected and mono-infected mice

Mice were infected with murine gammaherpesvirus by intranasal inoculation and after forty-five days, the same mice were challenged intranasally with sublethal dose of influenza virus.

Mice co-infected with MHV-68 and IAV were 55 days post MHV-68 infection / 10 days post infection (p.i.) with IAV very slim, had hyperaemic lungs, their intestines were empty and they had bristly fur. Forty-eight days later, all mice reached the standard weight without any clinical symptoms of the disease. At the 2nd interval, i.e. 6 days after IAV infection, the enlargement of thymuses of co-infected mice were observed in comparison to mice infected with only MHV-68. Mice infected with MHV-68 had 270 days p.i. hepatosplenomegaly and they were fatter than MHV-68 and IAV co-infected mice. It was evident that co-infected mice had less fat, there was no food in their stomach and intestine, and all of double infected mice had post-inflammatory necrotic lesion in lungs.

Co-infection increased the number of neutrophils in blood but did not affect the level of MHV-68 neutralizing antibodies

Based on comparison of differential blood counts (DBCs) of mice infected with MHV-68 alone and of co-infected mice we ascertained that the most significant differences were observed in levels of lymphocyte and neutrophil subpopulations of leuko-cytes. Percentage of lymphocytes in DBC after a single infection of mice with MHV-68 was by 20% higher in some time points in comparison to mice infected with influenza A virus only or to co-infected mice. Later after infection, on the 60th–225th day post infection with IAV, the number of lymphocytes in blood of co-infected mice was higher than in MHV-68 infected mice.

Based on DBCs, our results confirmed the importance of neutrophils function during influenza virus infection, as shown in the control group of mice. Throughout the acute IAV infection the amount of neutrophils in peripheral blood of co-infected mice increased 5-fold in comparison with herpes mono-infection, with maximum on 10th day after IAV infection. When mice cleared influenza infection, the percentage of neutrophils decreased dramatically (Fig. 1).

We didn´t observe any significant changes in titers of vi-rus-neutralizing antibodies against MHV-68 during persist-ent herpesvirus infection in comparison with the MHV-68 and IAV co-infection. Titers of virus-neutralizing antibodies against MHV-68 decreased proportionally to the length of primary herpes infection and ranged from 32 to 4.

Detection of herpesviral DNA in organs and reduction of ORF50 and ORF73 expression in the lungs and PECs

We demonstrated the presence of herpesviral DNA by nested PCR in all examined organs almost throughout the

Table 2. Percentage of lungs positive for ORF50 expression

		E1	E2	E3	E4	E5	E6
ORF50	H	50	50	25	25	0	25
	HI	0	0	25	0	0	0

Expression of ORF50 and ORF73 genes in lungs. E1-E6 = days of ex-amination (see Table 1). H-MHV-68 infected mice; HI-IAV and MHV-68 co-infected mice. Numbers represent percentage positive for ORF50 expression (0–100%).

Table 3. Percentage of lungs positive for ORF73 expression

		E1	E2	E3	E4	E5	E6
ORF73	H	50	50	50	25	0	0
	HI	25	0	0	25	0	0

Expression of ORF50 and ORF73 genes in lungs. E1-E6 = days of ex-amination (see Table 1). H-MHV-68 infected mice; HI-IAV and MHV-68 co-infected mice. Numbers represent percentage positive for ORF73 expression (0–100%).
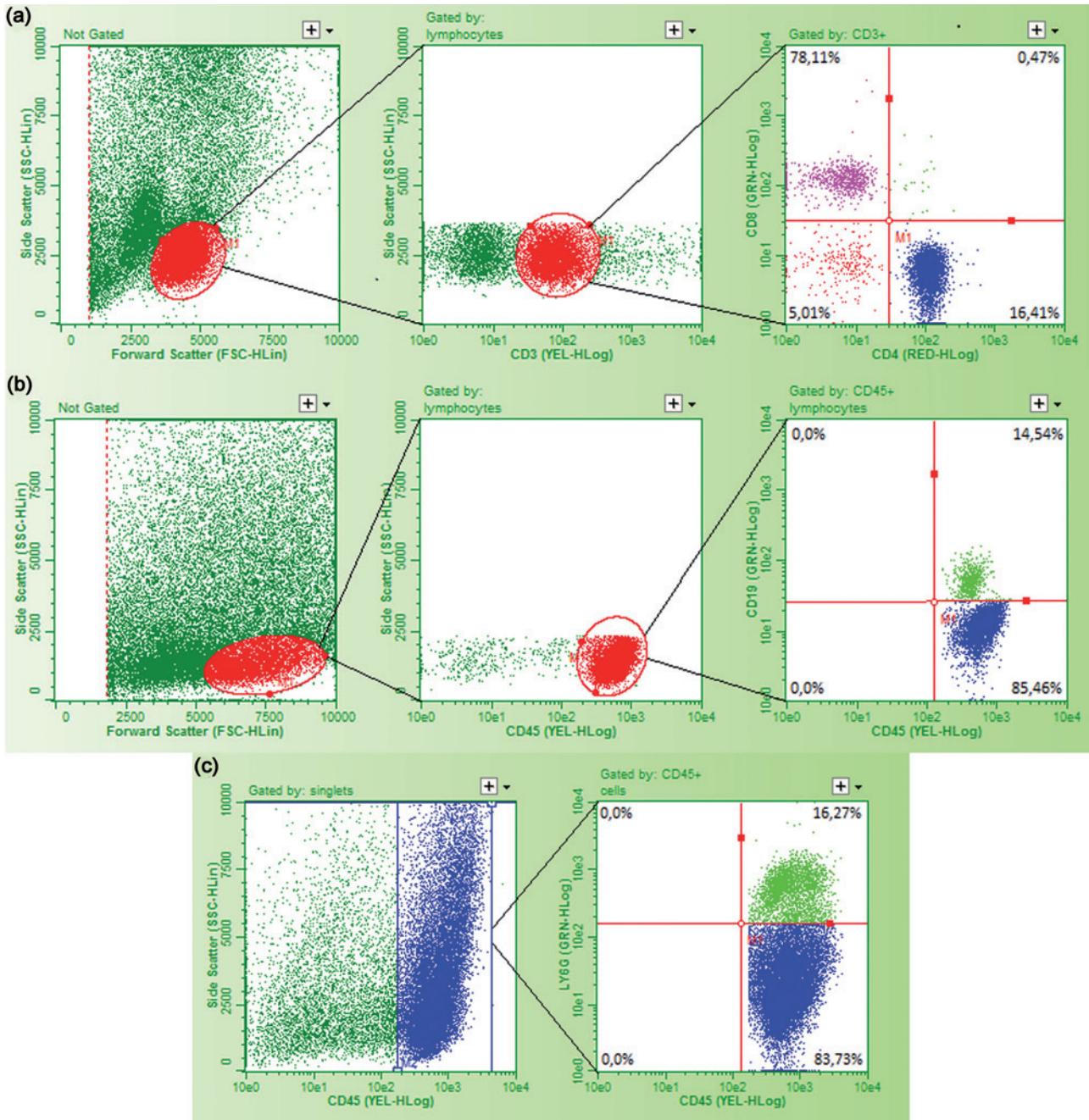
Fig. 2

Phenotypic analysis

(a) Phenotypic analysis of T-lymphocytes in lungs. Representative dot plot, as well as the gating strategy for identification and quantification of T-lymphocytes. Diverging lines indicate the sequence of gating. The gates for each dot plot are presented on the top of each box. The positive expression of markers compared to cells without antibody staining. Purple color represents the CD3+CD4+CD8- population whereas blue represents CD3+CD8+CD4- population. (b) Phenotypic analysis of B-lymphocytes in lungs. Representative dot plot, as well as the gating strategy for identification and quantification of B-lymphocytes. Bright light green color represents the CD19+CD45+ B cell population whereas blue represents CD19-CD45+ lymphocyte population. (c) Phenotypic analysis of neutrophils in lungs. Representative dot plot, as well as the gating strategy for identification and quantification of neutrophils. Bright light green color represents the Ly6G+CD45+ neutrophil population whereas blue represents Ly6G-CD45+ leukocyte population.

whole duration of the experimental infection with both viruses. We further examined the expression of ORF50 and ORF73 genes as markers of lytic infection and latency, respectively (Polčicová *et al.*, 2008). Samples from lungs,
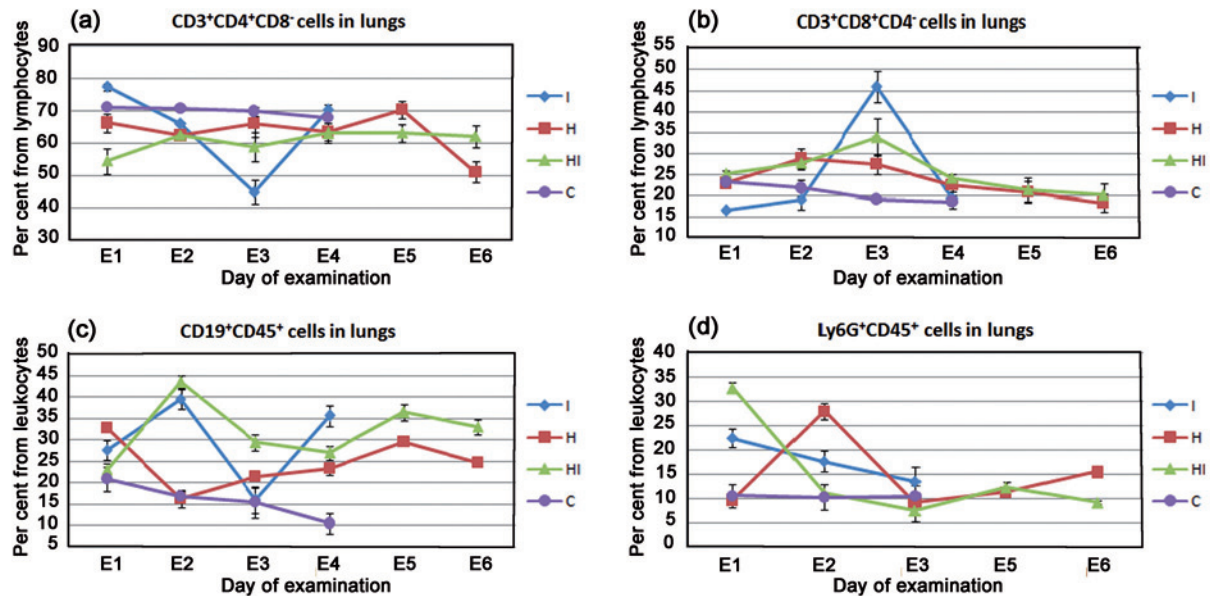
Fig. 3

Cellular profiles depict percentage of (a) CD3⁺CD4⁺CD8⁻, (b) CD3⁺CD8⁺CD4⁻, (c) CD19⁺CD45⁺ and (d) Ly6G⁺CD45⁺ cells in lungs collected from mice at the indicated time points after virus challenge and were determined using flow cytometry analysis
(a), (b) are determined as a percentage from lymphocytes population, (c), (d) as a percentage from leukocytes. E1-E6 = days of examination (see Table 1).

spleens, thymuses and PECs were obtained according to the scheme in (Table 1). Since MHV-68 establishes latency in lungs, we focused on detection of ORF50 and ORF73 expression during the secondary acute respiratory infection, in order to determine, whether influenza infection can affect herpesvirus reactivation from latency. We did not observe MHV-68 reactivation, but we recorded decreased expression of ORF50 and ORF73 during co-infection in lungs and this effect lasted during first two intervals for ORF50 and first three time points for ORF73 (Table 2, 3). The same phenomenon was detected in PECs, but MHV-68 expression was not altered in thymus and spleen. After influenza infection was overcome at the later time points (E4-E6) we did not see obvious differences between individual experimental groups of mice. What is responsible for the altered regulation of herpesvirus expression remains hypothetical.

Co-infection affected the number of leukocyte subpopulations in lungs but not in spleen

We observed that fifty-five days after MHV-68 and 10 days after IAV infection, the pool of CD4⁺/CD8⁻ T cells in lungs of double-infected mice decreased in comparison with the single herpesvirus infection (Fig. 2a, 3a). On the other hand, the pools of CD8⁺/CD4⁻ T and CD19⁺/CD45⁺ B cells in lungs of double-infected mice increased when compared to

single MHV-68 infection (Fig. 2b, 3b,c). Thus we found that CD8⁺ T cells expanded during the acute phase of influenza infection. This was in agreement with the effective elimination of vRNA of influenza virus from mouse lungs and with the decrease of titer of the infectious IAV in lungs (results not shown). It is known that CD8⁺ T cells are critical for the clearance of intracellular pathogens, including viruses and certain bacteria (Welsh *et al.*, 2010). We showed that ongoing gammaherpesvirus latency affected the number of cytotoxic T-lymphocytes during the acute influenza infection, which resulted in more effective clearance of IAV.

Examination of levels of neutrophils, showed a more rapid reduction of Ly6G⁺/CD45⁺ neutrophils in lungs of double-infected mice during the IAV co-infection, as compared to IAV mono-infected mice. The peak of number of neutrophils in lungs of co-infected mice was on the 3rd day after IAV infection and their percentage was about 48.8% lower than in mice infected with IAV only (Fig. 2c, 3d). On the other hand, the relative amount of Ly6G⁺/CD45⁺ neutrophils in the spleens of double infected mice, in comparison to mice solely infected with herpesvirus, was increased (Fig. 4d). No other notable differences between the investigated parameters were found in the spleen (Fig. 4a, b, c). Taken together, these results demonstrated that ongoing latent MHV-68 infection in co-infected mice affected the number of neutrophils during acute IAV infection.
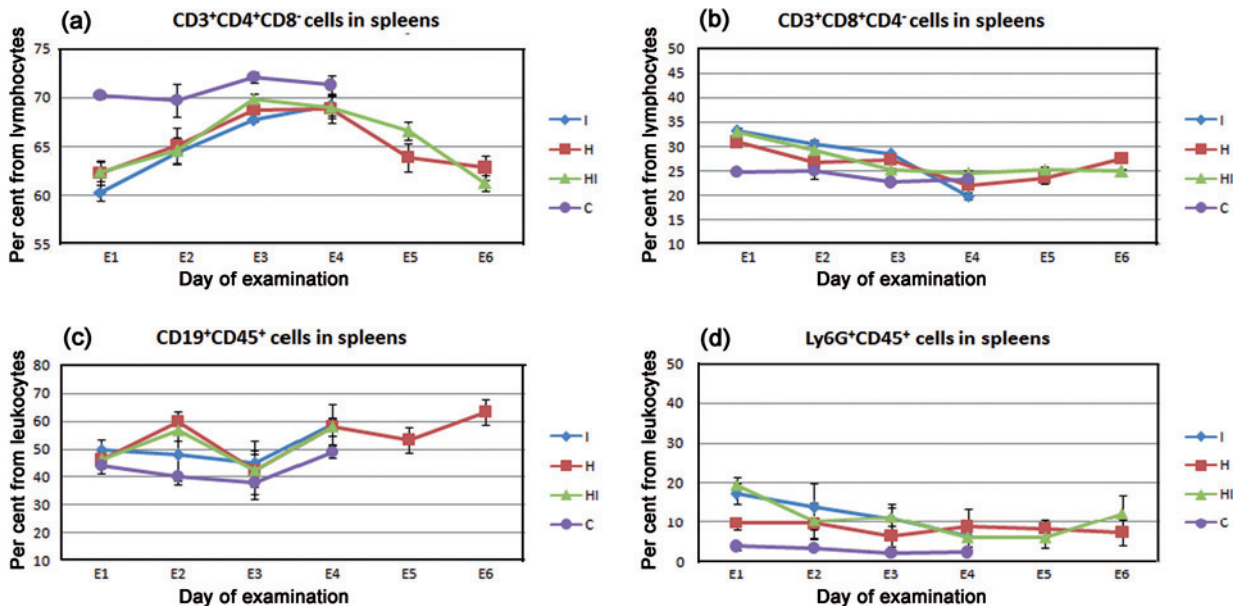
Fig. 4

Cellular profiles depict the percentage of (a) CD3⁺CD4⁺CD8⁻, (b) CD3⁺CD8⁺CD4⁻, (c) CD19⁺CD45⁺ and (d) Ly6G⁺CD45⁺ cells in spleens collected from mice at the indicated time points after virus challenge and were determined using flow cytometry analysis
(a), (b) are determined as a percentage from lymphocyte population, (c), (d) as a percentage from leukocytes. E1-E6 = days of examination (see Table 1).

Discussion

Simultaneous co-infection or super-infection of hosts, including humans, with multiple parasite species is a common event in the natural environment (Brooker *et al.*, 2012). Infections with different infecting agents can alter the immune response against each parasite and the abundance or persistence of the infection within the host. Such alteration of the immune response and change of infection course of the unrelated pathogen by the immune memory response caused by previously encountered pathogens is called heterologous immunity. It can lead to enhanced or diminished protective immunity and can alter immunopathology. In previous studies it was found, that infections with *Bacillus Calmette-Guérin* (BCG), IAV, LCMV, mouse cytomegalovirus and Pichinde virus all confer a level of protective immunity against vaccinia virus (Selin *et al.*, 1998; Mathurin *et al.*, 2009; Chen *et al.*, 2003).

Nearly 90% of humans are latently infected with herpesviruses that, after clearance of acute infection, produce little infectious virus and often cause no overt disease. Like the human gammaherpesviruses EBV and Kaposi´s sarcoma associated herpesvirus, MHV-68 establishes lifelong latency. Studies in this model system revealed that the latent MHV-68 can be reactivated *in vivo* by the secondary infection with parasitic helminths causing increased production of interleukin 4.

On the other hand, INF-γ inhibits MHV-68 replication and reactivation and controls persistent replication *in vivo* (Reese *et al.*, 2014). Persistent infections, however, can lead to chronic inflammation (Cárdenas-Mondragón *et al.*, 2013).

IAV-infected individuals often become co-infected with a bacterial pathogen and, consequently, morbidity and mortality significantly increase. The host plays a key role in better understanding of the way of interaction of these pathogens with each other (Smith *et al.*, 2013).

Three possible points of view can be discussed regarding the triangle: host – persistent infection and the acute infection. First consideration is from the view of the host, whose aim is to suppress the acute infection, restrain the latent infection and possible reactivation and constrain the immune response to avoid the harmful immunopathology. The second interpretation is from point of view of latent virus that seeks (needs) to ensure such conditions, which allow it to persist in the host without its lethal damage (Fujinami *et al.*, 2006). And the last one is the viewpoint of the virus causing acute infection, which aim is to replicate effectively and to spread to another hosts. There is no comprehensive understanding of the immune responses during co-infections and it is unclear how the systemic and local defense mechanisms interact (Thakar *et al.*, 2012).

In the present study we focused mainly on the second view mentioned above. We investigated the effect of the

acute influenza infection on the course of long-term chronic herpesviral infection and its capability to influence reactivation of the latent virus. We performed our research using mouse *in vivo* model with maintained persistent infection of MHV-68 followed by the acute infection with IAV. Several clinical symptoms were observed in co-infected mice during the chronic infection with MHV-68.

In all investigated parameters throughout the duration of co-infection we observed the same trend 55 days p.i. with MHV-68 / 10 days p.i. with IAV mice, namely the ability to overcome double viral load and all parameters monitored in co-infected mice leveled with values of herpes mono-infected animals.

As for acute influenza infection, we demonstrated that CD8^{+} T cells expanded and cleared IAV infection earlier. This observation is in agreement with previously published observations that ongoing gammaherpesvirus latency affects the number of memory cytotoxic T-lymphocytes during the acute influenza infection (Saito *et al.*, 2013). The increase of CD8^{+} T cells occurs to the detriment of the number of the CD4^{+} T-lymphocytes. CD8^{+} T cells are also considered a key player in the prevention of herpesviral reactivation during an acute heterologous viral infection (Sharma and Thomas, 2014). Taken together, we can conclude that the number of lymphocytes in peripheral blood, as well as CD8^{+}/CD4^{-} T cells and CD19^{+}/CD45^{+} B cells in lungs of co-infected animals were increased in comparison with mono-infected mice which can lead to higher production of IFN-γ and antibodies, resulting in stimulation of the immune system. Thus, it can explain a more effective suppression of IAV replication and prevention of herpesvirus reactivation from latency.

An important role of CD3^{+}/CD4^{-}/CD8^{-} T cells in lungs of co-infected mice during the first days of acute IAV infection can be also assumed, since on day 3 after IAV infection their percentage was 2.5-fold or 2-fold higher in co-infected animals than in IAV mono-infected mice or in MHV-68 infected mice, respectively (data not shown). This type of cells is considered a major responding T cell subset in lungs of mice infected by various viruses or intracellular bacteria. They are also more rapid and effective producers of IL-17 in early stages of infection than CD4^{+} T cells (Cowley *et al.*, 2010; Lin *et al.*, 2009; D'Acquisto and Crompton, 2011).

Significant increase in the amount of neutrophils in peripheral blood of co-infected mice and notable elevation of Ly6G+/CD45+ neutrophils in lungs of double-infected mice in comparison with the single herpes-infected mice were observed at the 3rd day of the acute influenza infection. Rate of neutrophils during co-infection had similar trend as seen in IAV mono-infected mice except for lungs, where during early stages after secondary IAV infection the number of neutrophils was markedly elevated but dropped rapidly before 6th day p.i. with IAV when compared to single IAV infection. In contrast, the pool of Ly6G^{+}/CD45^{+} neutrophils in the spleen

of double-infected mice had increased just mildly compared to the single herpesvirus infection. These results demonstrate that ongoing latent MHV-68 infection in co-infected mice affect the number of neutrophils in tissue/organ-dependent manner during acute IAV infection. Neutrophils are responsible for the pathological inflammatory infiltration and the necrosis. Many studies aiming to identify the roles of different subpopulation of leukocytes during the influenza infection were performed. In addition to macrophages and NK cells, neutrophils are also recruited into the lungs during influenza virus infection and play an important role in the immunopathology (Xu *et al.*, 2006; Deng *et al.*, 2010; Kostolanský *et al.*, 2013). However, the role of neutrophils in influenza virus infection seems to be ambivalent. Two reports showed that the depletion of neutrophils before influenza virus infection resulted in higher mortality rates in mice (Ito *et al.*, 2011; Fujisawa 2008), while other reports showed that increased numbers of neutrophils in lungs during influenza infection were associated with severe pneumonia and high mortality (Saito *et al.*, 2013; Kostolanský *et al.*, 2013; Perrone *et al.*, 2008). The course of infection and the role of neutrophils within it appear to be dependent on the virus virulence. Our observations correspond to previous studies demonstrating that infection by influenza virus with mild and intermediate virulence leads to a transient weight loss of infected mice but they did not succumb to the infection. Pathogenesis is associated with the lung injury, however the recovery of the lung tissue and the clearance of the virus are not connected with an increased number of the neutrophils in the lungs and the alternative immune defense is sufficient to overcome the infection (Tate *et al.*, 2011). The study of Saito *et al.* revealed lower numbers of neutrophils in lungs of MHV-68 and influenza virus co-infected mice, which is interpreted as a regulated neutrophil influx by the activated macrophages (Saito *et al.*, 2013).

Titer of virus-neutralization antibodies against MHV-68 does not increase proportionally to the length of MHV-68 infection, but its level decreased proportionally with the time after the secondary influenza infection. Significantly decreased titer of antibodies against MHV-68 ranged from 4 to 32. The maximum production of antibodies against MHV-68 was detected at the 1st time point, which could be explained by the climax of the acute secondary influenza infection ongoing at that time.

Monitoring of the expression of ORF50 and ORF73 genes, the markers of lytic and latent phase of infection showed that their expression varied in individual samples and at various time points, despite the detection of herpesviral DNA almost in every sample throughout the duration of the experimental infection. At some intervals the difference between the expression of ORF50 and/or ORF73 in lungs of co-infected mice and herpes-infected mice was 50%. It is unquestionable that the secondary influenza infection did not cause reactiva-

tion of latent MHV-68, but surprisingly it led to a suppressed expression of selected MHV-68 genes. This can be a result of cap-snatching mechanism, which influenza virus uses for its replication and which leads to degradation of mRNAs other than influenza virus ones. This process can affect the MHV-68 gene expression in the cells that can be infected with both viruses, such as macrophages (alveolar and peritoneal) and epithelial cells (Rodger and Mims, 1981; Xie *et al.*, 2009; Shih and Krug, 1996). It can result in the decreased number of MHV-68 transcripts to almost undetectable level. The inhibition of the ORF50 expression, which is the main trans-activator of the lytic genes, crucial for the reactivation from latency, can contribute to the attenuated reactivation of MHV-68 and its persistence in silent latent state (Virgin *et al.*, 1997; Wu *et al.*, 2000; Damania *et al.*, 2004; Ebrahimi *et al.*, 2003). Besides a transcript degradation, IAV acute infection is responsible for the apoptosis of a portion of host macrophages and lymphocytes what can also lead to a decrease in the number of the MHV-68 transcripts and to the reduction of its latent reservoir (Nichols *et al.*, 2001). Prevention of reactivation is also ensured, as was mentioned above, by the CD8^{+} T cells, the production of cytokines, mainly IFN-γ and the antibodies (Reese *et al.*, 2014; Kim *et al.*, 2002).

Based on our results, it is obvious that the concurrent latent herpesvirus infection and acute influenza co-infection affect numerous parameters of the host immune response. IAV infection did not lead to the reactivation of the latent MHV-68 and in cooperation with the host immune system suppressed the expression of the herpesvirus genes to a nearly undetectable level. This phenomenon could be observed mainly in lungs and PECs. Similarly, in other studies dealing with co-infection of MHV-68 and other pathogens, different course of disease, in comparison with the individual infections, was detected. Mice latently infected with MHV-68 followed by acute malaria did not develop any signs of infection during the experiment (Haque *et al.*, 2004). In other studies it has been reported that MHV-68 latency modulates the host immune response to influenza A virus (Saito *et al.*, 2013).

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