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Morphofunctional status and the role of mononuclear phagocyte system lung compartment in the pathogenesis of influenza A (H5N1) in mammals

Anna V. Kovner, Oxana V. Potapova, Vyacheslav A. Shkurupy, Alexander M. Shestopalov

FSBI Research Center of Clinical and Experimental Medicine, Siberian Branch of the Russian Academy of Medical Sciences, Novosibirsk, Russia
Email: anya.kovner@gmail.com

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ABSTRACT

Influenza and other respiratory viral infections account for 80% - 90% of infectious pathologies. Influenza A (H5N1) virus has an apparent pneumotropism, and therefore the lung compartment of mononuclear phagocyte system plays an important role in antiviral immunity. Lung macrophages are active phagocytes expressing variety of antiviral factors. The investigation of morphofunctional status of lung macrophages and evaluation of their role in mammal antiviral response in a mouse model were performed within the study. Methods: Light microscopy using standard hematoxylin-eosin, and Van-Gizon's picrofuchsin staining. Immunohistochemistry using influenza A antigen marker specific primary antibodies, myeloperoxidase, cathepsin D, lysozyme, NO synthase, pro-inflammatory cytokines, cells of CD68 macrophage lineage, PCNA proliferative activity. Morphometric and statistical analysis. Results: Influenza A virus antigen was detected in lung macrophages starting from day 1 to day 14 of infection which corresponds with the beginning of convalescence and may be suggestive of prolonged persistence of virus. On the one hand, the cytopathic effects of the virus lead to lung macrophages death mainly via apoptosis through activation of caspase cascade, including caspase-3 and caspase-9. On the other hand, the observed activation of PCNA proliferation marker, perhaps, allows to support the pool of lung macrophages not only by their recruitment from bone marrow but also by their proliferation *in situ*. The increase of mononuclear phagocyte system cells expressing antiviral factors depended on the stage of infection. In the early stage, there was an increase of number of cells expressing lysozyme, myeloperoxidase, cathepsin D, endothelial NO synthase (eNOS) followed by the increase of number of macrophages expressing inducible NO synthase (iNOS), pro-inflammatory cytokines and interleukins.

Keywords: The Influenza A (H5N1) Virus; Macrophages of Lungs; Cathepsin D; Myeloperoxidase; Lysozyme; iNOS; eNOS; TNF-α; IL-6; PCNA; Cell Death

1. INTRODUCTION

Influenza and other respiratory viral infections are the most wide-scale and account for 80% - 90% of infectious pathologies. The ability of influenza viruses to cause frequent epidemics and even pandemics is a determining feature of diseases that cause problems of global importance. From 5% to 20% of population suffer from the disease during epidemic. Owing to changing of virus properties (particularly contagiousness) it is not infrequent that every second person can fall ill during pandemic [1]. Constant "evolution" of influenza A (H5N1) virus (one of the most pathogenic influenza virus), the possibility of their reassortment with human population adapted influenza A virus strains and multiresistance to available antivirals are the pandemicity factors. In spite of high priority of influenza challenge, the number of aspects of the disease pathogenesis in mammals and human remains insufficiently explored [2].

It is known that macrophages are the main biological factor of virus elimination from body. Aerogenic rout of infection determines an important role of lung compartment of mononuclear phagocyte system in development of influenza A (H5N1) virus infection.

The most numerous lung macrophage population is located on the surface of alveolar epithelium [3]. Alveolar macrophage is a central and specialized element of mononuclear phagocyte system cell population and plays

a leading role in case of influenza A (H5N1) infection [4]. However, the appearance of new influenza A (H5N1) virus strains also imply the possibility of the whole mononuclear phagocyte system (and particularly it's lung compartment) response change.

Alveolar macrophages phagocyte viral particles as well as apoptotic and necrobiotic cells thereby protect body from damages induced by influenza A (H5N1) viruses [5]. However, viral replication in macrophages results in their death and more severe disease in case of mammal infection with influenza A (H5N1) viruses [6].

Thanks to advanced lysosomal apparatus, macrophages can exhibit antiviral activity through secretion of lysosomal enzymes (myeloperoxidase, lysozyme, cathepsin D, acid hydrolase, etc.) [7], more or less successful capture and digestion of microorganisms in vacuolar apparatus, as these enzymes perform intracellular breakdown of viral protein structures.

Thus, due to secretion of antiviral factors, the phagocytosis of infected cells and antigen-presenting for other immune cells of host, macrophages are the key element of influenza A (H5N1) pathogenesis.

Study objective—to investigate the morphofunctional and quantitative changes of macrophages in lungs of C57Bl/6 mice and their role in the process of shaping of antiviral response to infection with A/goose/Krasnoozerskoye/627/05 (H5N1) avian influenza virus strain recently reported in Russia.

2. MATERIALS AND METHODS

The work has been done on 90 2-month-old C57Bl/6 male mice with body weight of 20 - 25 g (laboratory animal nursery of Scientific Research Institute of Clinical Immunology SB RAMS. Novosibirsk, Russia) infected with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus, isolated from the lung of goose which died during avian influenza outbreak in Krasnoozerskoye village, Novosibirsk region, Russia in September 2005. The choice of this strain is determined by its high pathogenicity and an ability to replicate in many organs of mammals (lungs, liver, kidneys, spleen, brain) [8]. Animals were intranasally infected with 5 MLD_{50} dose. Intact animals (15 mice) were used as controls. Animals were housed in standard conditions with free access to food and water. Experimental works with influenza A (H5N1) viruses were conducted at the premises of laboratory in Department of Zoonotic Infections and Influenza, FBRI SRC VB VECTOR, Novosibirsk.

Animals were taken out of the experiment through dislocation of vertebrae in cervical spine. Lungs obtained for investigation on 1, 3, 6, 10, and 14 days after infection were the object of study.

After fixation in 10% neutral formalin solution the obtained material was dehydrated using series of ethanols with growing concentrations and xylols and embedded in synthetic paraffin mixture "HISTOMIX" (BioVitrum, Russia) in order to perform light-optical investigation. Sections of 3.5 μm thickness were made on microtome ("MICROM", Germany). Sections were stained with hematoxylin-eosin, and picrofuchsin by Van-Gizon's technique.

Immunohistochemical (IHC) analysis was performed using indirect streptavidin-peroxidase method with specific primary antibodies against influenza A (Inf A) antigen (Inf A/FITC ("Abcam")), lysosomal enzymes (Cathepsin D ("DBS"), Myeloperoxidase ("DBS"), Lysozyme ("DBS")); NO synthase (iNOS ("Spring BioScience"), eNOS ("Abcam")); pro-inflammatory cytokines (TNF-α ("DBS"), IL-6 ("Novocastra")); macrophage marker (CD68 ("DBS")); nuclear marker of proliferative activity (PCNA ("Novocastra")), caspases: (Caspase-3, Caspase-9 ("Abcam")).

In order to perform the immunohistochemical investigation, 3 μm lung sections were dewaxed, dehydrated, epitope-retrieved in citrate buffer solution in 700 W microwave oven during 20 - 35 minutes. Endogenous peroxidase were being blocked for 5 minutes after single wash in distilled water and phosphate buffer solution (PBS). The time of exposition to primary antibodies at 37˚C was 30 - 45 minutes. Sections were incubated with streptavidin-peroxidase complex and DAB substrate and finished staining with Mayer's hematoxylin. Sections were dehydrated using ethanols with growing concentrations and xylol, mounted with synthetic mounting media "Bio Mount" (BioVitrum, Russia) and placed under cover glasses.

The analysis of histologic specimens was performed using AxioImager A1 microscope with AxioCam MRc camera (Carl Zeiss, Germany). Morphometry study of structural elements was conducted using graticule with 100 3.64×10^5 μm^2 points (to determine the numerical density (NAi)) [9] and AxioVision (rel. 4.12.) software instruments.

The average values of investigated parameters were determined using "Statistica" standard software package. The significance of differences between average values was determined using Student t-test and the Pearson correlation analysis was performed. Differences were considered significant at $p < 0.05$.

3. STUDY RESULTS

It has been shown earlier that macrophages are one of main target cells for influenza A viruses [10-12]. Influenza viruses were identified in lung macrophages as early as one day after infection when studying mice lung samples using immunohistochemical analysis based on Inf A antibody against influenza A (H5N1) virus antigen. The number of macrophages expressing influenza A (H5N1) virus antigen grew by day 3 with a subsequent 3.4-fold decrease by day 14 (**Figures 1** and **2**).

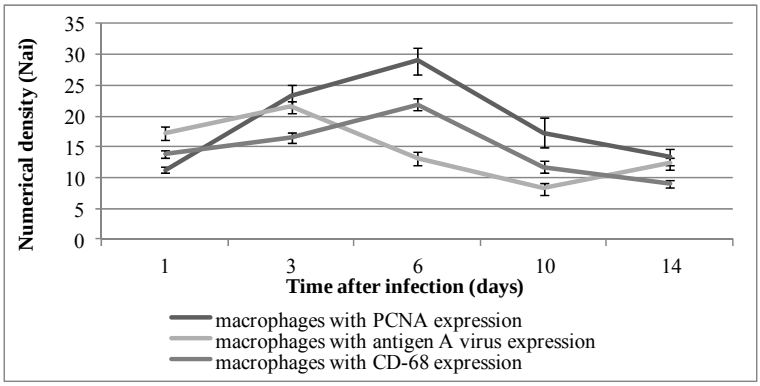

Figure 1. Estimation result for numerical density of macrophages expressing PCNA, influenza A virus antigen and CD68 in mice lungs infected with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus.

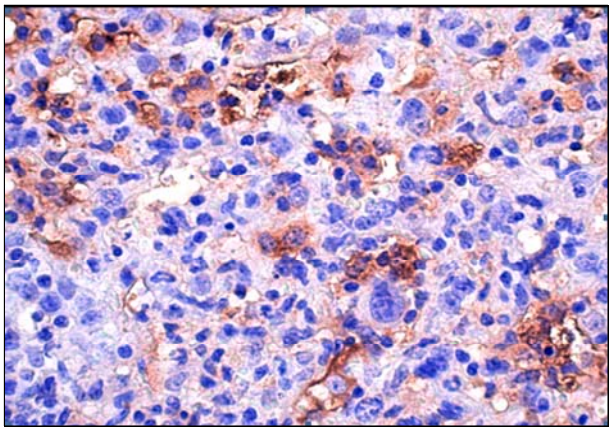

Figure 2. Influenza A virus antigen expression by macrophages of the lung of mice infected with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus. Sixth day after infection. Immunohistochemical analysis. Magnitude ×630.

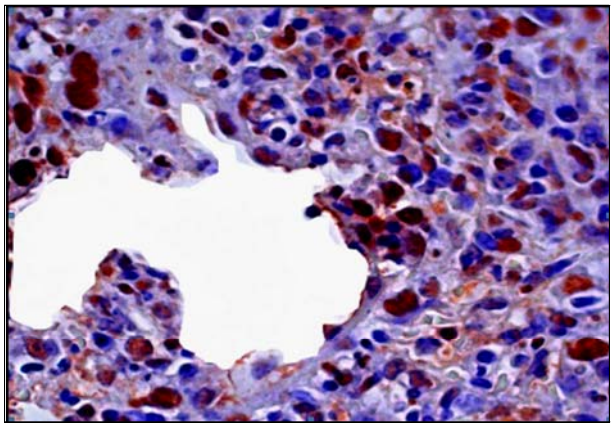

Figure 3. Lung fragment of mice infected with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus. PCNA expression by macrophages of mice infected with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus. Tenth day of experiment. Immunohistochemical analysis. Magnitude ×630.

Accumulation of large number of monocytes and macrophages in lung interstice, peribronchially and inside alveoli is suggestive of activation of cellular component of immunity in the early stages of viral infection [13]. The number of CD68 macrophages has increased 1.4-fold from 1 to 6 day of experiment and exceed the same parameter of intact animals 4.7-fold on day 3 after infection. The number of macrophages has decreased by day 6 after infection (**Figure 1**).

The activity of lung macrophage proliferation was evaluated based on expression of nuclear marker of proliferative activity—PCNA (**Figures 1** and **3**). The increase of number of lung macrophages expressing this factor in mice infected with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus was observed from 1 to 6 day of experiment. It was a 2.6-fold increase followed by decrease by day 14 of infection. This could be related to the death of macrophages due to viral persistence inside them and to the insufficient arrival of macrophage precursors from bone marrow (**Figure 1**).

It was established during the study that destructive changes, presented by necrotic and apoptotic foci, developed in lungs after mice infection with influenza A (H5N1) virus. The numerical density (Nai) of lung macrophages expressing caspase-3 and caspase-9 was investigated in order to study the scales of apoptosis. The scale of macrophage death through apoptosis was larger than the scale of necrosis in all periods of the study (**Table 1**), which, perhaps, could be caused by the cytopathic effect of viruses [14].

The number of macrophages expressing caspase-9 marker which is suggestive of the beginning of apoptotic changes without visible morphological manifestations were maximum on day 3 of experiment with a subsequent decrease by day 14 of infection (**Figure 4**, **Table 1**). The number of caspase-3 positive lung macrophages in C57Bl/6 mice was maximum on day 6 with a 30% increase and it decreased by 14 day (**Figures 4** and **5**, **Table 1**).

The obtained data suggest that infection and persis-

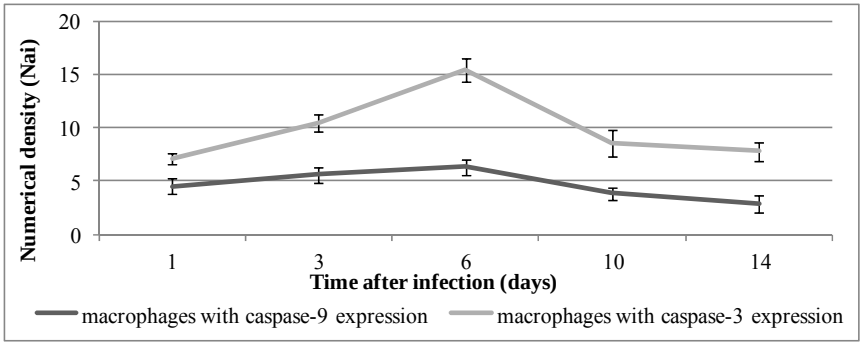

Figure 4. Estimation result for numerical density of macrophages expressing caspase-3 and caspase-9 in lung of mice infected with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus.

Table 1. Numerical density of C57Bl/6 mice lung macrophages in the state of cell destruction after infection with A/goose/ Krasnoozerskoye/627/05 (H5N1) influenza virus (M ± m).

Objects and parameters of the study	Time after infection, days	Macrophages
Numerical density of macrophages in destruction state, (Nai)	1	16.8 ± 0.85
	3	19.8 ± 0.83[b]
	6	26.4 ± 1.83[b]
	10	17.4 ± 1.45[b]
	14	15.8 ± 0.65[b]
Of them:		
Percent of macrophages expressing caspase-9, (%)	1	26.93 ± 1.55
	3	28.2 ± 1.22[b]
	6	23.8 ± 1.46[b]
	10	21.9 ± 1.19
	14	17.8 ± 0.98[b]
Percent of macrophages expressing caspase-3, (%)	1	42.4 ± 1.53
	3	52.8 ± 2.25[b]
	6	58.4 ± 1.73[b]
	10	49.2 ± 2.2[b]
	14	49.3 ± 1.4

[b]The significance of difference of considered parameter values as compared with previous period of investigation.

tence of influenza A (H5N1) viruses in mice lung macrophages initiates not only the processes of cell destruction but also the processes of proliferation and that is suggestive of contribution of this factor in cell number maintenance *in situ*.

It is known that early antiviral protection of body is realized by oxygen-independent nonspecific factors, primarily lysozyme and cathepsin D [15].

The increase of lung macrophages functional activity in this experiment was registered as early as on the first day after mice infection, judged by macrophage expression of intracellular lysosomal enzymes. The number of lung macrophages expressing lysozyme marker was

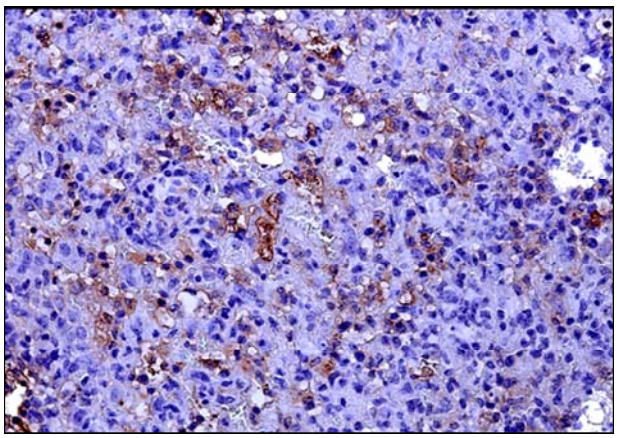

Figure 5. Lung fragment of mice infected with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus. Caspase-3 expression by alveolocytes, macrophages and endotheliocytes of lung vessels of mice of mice infected with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus. Tenth day of experiment. Immunohistochemical analysis. Magnitude ×630.

maximum at the first day of experiment and exceeded that of control group 6.6-fold (**Table 2**, **Figure 6**), which makes it possible to rate lysozyme as "fast response" enzyme. The number of these cells decreased by day 10 of experiment (**Table 2**), but a repeated increase of lysozyme expressing macrophage number was registered by day 14 of experiment, which can be associated with continued lung persistence of viruses and, perhaps, with their intercellular circulation and macrophage clearing function with respect to the products of lung cell necrosis and apoptosis.

The other investigated factor of oxygen-independent system of antiviral protection was cathepsin D, which maximum expression in mice lung macrophages were registered at day 1 and 6 after infection with a decrease by day 14 of experiment (**Table 2**).

The activation of factors of oxygen-dependent system of antiviral protection, among which myeloperoxidase and NO synthase are the most important, was observed 3

Table 2. Numerical density (Nai) investigation results for lung macrophages, expressing intracellular lysosomal enzymes after infection with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus (M ± m).

Objects and parameters of the study	Time after infection, days	C57Bl/6 mice	
		Intact	Infected
Numerical density of macrophages in destruction state, (Nai)	1	3.7 ± 0.55	24.2 ± 1.42[a]
	3		16.3 ± 0.73[ab]
	6		6.7 ± 0.71[ab]
	10		3.9 ± 0.35[b]
	14		5.8 ± 0.83[ab]
Percent of macrophages expressing caspase-9, (%)	1	4.9 ± 0.84	14.6 ± 1.02[a]
	3		6.7 ± 0.92[ab]
	6		17.6 ± 1.89[ab]
	10		14.1 ± 0.42[ab]
	14		8.5 ± 1.02[ab]
Percent of macrophages expressing caspase-3, (%)	1	3.6 ± 0.63	20.8 ± 2.00[a]
	3		25.6 ± 0.63[ab]
	6		18.8 ± 0.52[ab]
	10		9.2 ± 0.7[ab]
	14		9.5 ± 0.63[a]

[a]The significance of difference of average values as compared with that of control; [b]The significance of difference of considered parameter values as compared with previous period of investigation.

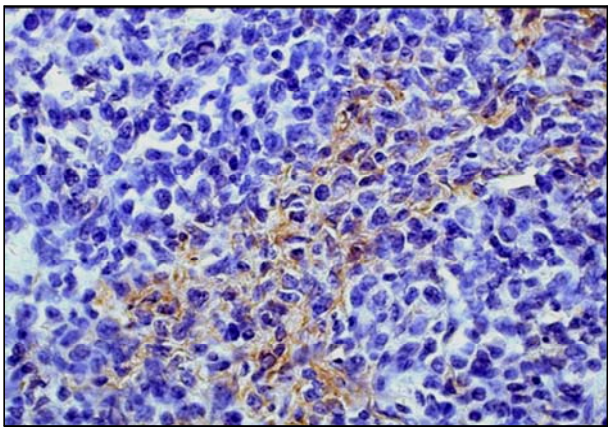

Figure 6. Lung fragment of mice infected with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus. Lysozyme expression by alveolocytes and macrophages of mice infected with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus. Tenth day of experiment. Immunohistochemical analysis. Magnitude ×630.

days after infection. The number of lung macrophages expressing myeloperoxidase marker was maximum on day 3 of experiment and exceeded control value almost 7-fold (**Table 2**).

Besides myeloperoxidase, the NO nitrogen oxide, activated through endothelial and inducible NO-synthase, bulk large among oxygen-dependent mechanisms. When C57Bl/6 mice were infected with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus, the number of lung macrophages expressing iNOS increased 6.9-fold from 1 to 9 day of experiment with a subsequent 3.4-fold decrease by 14 day of experiment (**Figure 7**).

The number of macrophages expressing eNOS was maximum on the first day of experiment and 4.1 times bigger than that of control (**Figure 7**). The concentration of cells expressing eNOS decreased by 10 and 14 day of experiment but exceed the value of the same parameter for intact animals (**Figure 7**). The expression of eNOS was maximum on the early stages of mice response to viral infection and was higher than expression of iNOS, which increased as the expression of eNOS decreased (**Figure 7**).

Owing to hypersecretion of pro-inflammatory cytokines, among which IL-6 and TNF-α are considered having an important role, the immunopathogenesis of influenza caused by the influenza A (H5N1) virus is manifested primarily in hypercytokinemia [16]. Unlike seasonal strains of influenza viruses, influenza A (H5N1) viruses are potent TNF-α and IL-6 inductors *in vivo* as well as *in vitro* [8,17].

As early as at the first day of experiment the number of lung macrophages expressing IL-6 marker exceeded that of intact animals 4.1-fold having it's peak on day 3 after infection of mice and by 14 day it exceeded the same parameter in intact mice 3.2-fold (**Figures 8** and **9**).

The number of lung macrophages expressing TNF-α marker was maximum on day 6 of experiment. The number of TNF-α-positive macrophages decreased by day 14 of infection still remaining high in comparison with control group (**Figure 8**). The number changing pattern of cells expressing both cytokines (**Figure 8**) indicates the possibility of lung damaging and inflammation processes beyond the period of this experiment.

4. CONCLUSIONS

Inf A virus antigen was observed in lung macrophages since the first day of experiment during the immunohistochemical study of A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus topology in the lungs of infected C57Bl/6 mice. The interaction between macrophages and viral particles and is mediated by Fc-receptors [18] and it is accompanied by redundant secretion of pro-inflammatory cytokines and chemokynes resulting in a large-scale secondary alteration of lungs.

The cytopathic effects of influenza A (H5N1) virus lead to elimination of macrophages in lungs mainly via caspase-dependant apoptosis through activation of initiator caspase-9 and effector caspase-3. The detected expression of PCNA nuclear marker in lung macrophages is suggestive of a certain contribution of macrophage proliferation *in situ*—as a mechanism of lung

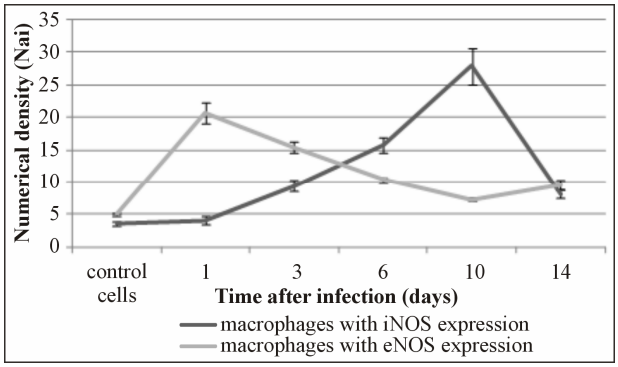

Figure 7. Estimation result for numerical density of macrophages expressing iNOS and eNOS in mice lungs, infected with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus.

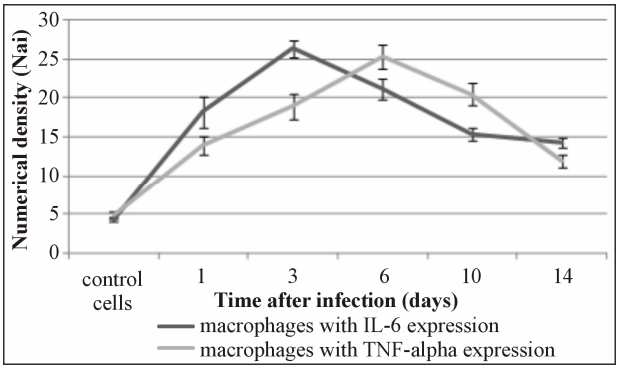

Figure 8. Estimation result for numerical density of macrophages expressing IL-6 and TNF-α in mice lungs, infected with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus.

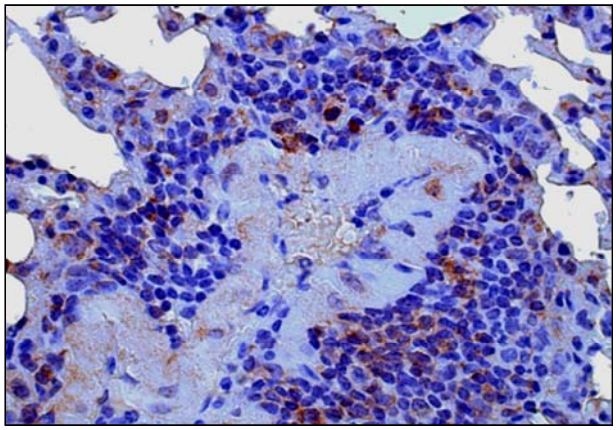

Figure 9. Lung fragment of mice infected with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus. IL-6 expression by alveolocytes, macrophages, and endotheliocytes of lung vessels of mice infected with A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus. Third day after infection. Immunohistochemical analysis. Magnitude ×630.

macrophage number maintenance besides recruitment of blood monocyte of bone-marrow origin [19]. On the other hand, the DNA synthesis activation in lung macrophages of C57Bl/6 mice may be associated with increase of their synthetic and phagocytic activity suggesting of phenotypic differentiation of juvenile cells of mononuclear phagocyte system recruited from bone marrow.

Thus, lung macrophages play an important role in antiviral protection through secretion of different factors that "alternate" depending on the stage of infection. However, an efficient replication of A/goose/Krasnoozerskoye/627/05 (H5N1) influenza virus in lung cells of infected C57Bl/6 mice, the significant increase of pro-inflammatory cytokine and NO synthase secretion by lung macrophages results in acute phase response and accumulation of destructive changes in lung tissue and inflammatory manifestations.

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